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The role of histone deacetylase 1 in neuroregeneration in the zebrafish spinal cord

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Declaration

I declare that this thesis has been completed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgement, the work presented is entirely my own.

Tess McCann

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Lay summary

The central nervous system (CNS) is made up of the brain and spinal cord. It contains specialised cells called neurons that transmit signals to each other and are responsible for many functions such as movement. In the CNS of mammals including humans, repair of the brain and spinal cord is limited after an injury with recovery often not achieved. Zebrafish however display high repair capability after an injury, for example of the spinal cord, such that they can regain swimming function after complete paralysis.

In development the progenitor cells in the spinal cord make the different neurons. When adulthood is reached these cells go into an inactive state and no longer produce new cells. After an injury these progenitors must become active again to make the new cells to replace what has been lost in the injury. After an injury the mammalian spinal cord progenitors fail to make neurons, however, the zebrafish progenitors can. We study the repair process in the zebrafish to understand the mechanisms that allow them to repair successfully. This may help inform mechanisms that could help improve repair in the mammalian spinal cord.

After an injury the progenitors will receive many different external signals like those from the inflammation associated with spinal cord injury. Factors inside the progenitor also change to re-program these cells for repair. In my project I analysed an internal factor called Hdac1. The role of Hdac1 in the cell is to change how the instructions that tell the cell how to behave are read. I made genetic tools to increase or decrease the activity of Hdac1. I found that decreasing Hdac1 led to a reduction in the numbers of neurons made after a spinal cord injury. This indicates that Hdac1 has a positive role in repair in zebrafish. I also found that increasing Hdac1 in the progenitor cell, without injuring the spinal cord, could trigger them to become more active.

In conclusion, Hdac1 in the progenitor was found to have a positive role in spinal cord repair after an injury and may be one of the internal changes that occurs to move them out of the inactive state. Future work will look into what are the instructions that Hdac1 has altered to enable this repair process. Ultimately understanding repair in zebrafish may aid the finding of new therapeutic strategies for spinal cord injury in mammals.

Abstract

In contrast to mammals, zebrafish show high regenerative capability after injury to the central nervous system (CNS). For example, after spinal cord injury zebrafish produce new neurons at the site of injury and extend axons back across the site to reform connections. This successful repair leads to functional recovery. The regenerative neurogenesis is performed by endogenous ventricular progenitor cells termed ependymo-radial glial (ERGs). In homeostatic conditions the ERGs are in a quiescent state but after the injury are triggered to proliferate and differentiate into the lost cell types of the zebrafish spinal cord.

Previous work in the group has studied a range of different external signals such as Notch ligands and Sonic hedgehog that influence the ERGs during this repair process. The downstream mechanisms within the ERGs that are altered due to these signals are unclear. In my thesis I investigated one of the intrinsic changes within the ERGs that is involved. The epigenetic regulator Histone deacetylase 1 (Hdac1) was an attractive target to study as it had been found to regulate the activity of the above external signalling pathways and to promote the expression of transcription factors involved in developmental neurogenesis in zebrafish. Hdac1 mRNA expression is increased in the ERGs after a spinal cord injury. I hypothesised that the increased Hdac1 activity in the ERGs could be a mechanism that facilitates the integration of the different extrinsic pathways which leads to successful regeneration in zebrafish.

To investigate the role of Hdac1 during regenerative neurogenesis in the lesioned spinal cord, I generated genetic tools that would allow cell-specific manipulations of Hdac1 in the ERGs. This was necessary as pharmacological approaches were limited in two respects. Firstly, since Hdac1 is expressed in all cells, drugs that inhibit Hdac1 would have global effects. This is an important consideration as Hdac1 may have different roles depending on the cell type in question. For example, inhibitors cause immunosuppression and the immune response is an important trigger for regenerative neurogenesis. Hence, effects on neurogenesis may be indirect. Secondly there are no drug compounds that can activate Hdac1 directly, preventing gain of function experiments. Therefore, I generated two new transgenic zebrafish lines that could decrease or increase Hdac1 only within the progenitor cells. I used the Tet-On system to drive conditional expression of a dominant negative form of Hdac1 (*dnhdac1*) or wildtype *hdac1* in the ERGs. I confirmed that these lines were specific to ERGs and could functionally alter Hdac1 levels.

I used these new transgenic lines to test the role of Hdac1 during neuroregeneration in the lesioned spinal cord of zebrafish. I assessed regenerative neurogenesis after spinal cord injuries in both larval and adult zebrafish. I found that expression of *dnhdac1* decreased regenerative neurogenesis, while expression of wildtype *hdac1* did not further boost regeneration.

To test whether a decrease in acetylation levels could play a role in stimulating the ERGs to leave their quiescent state, I used the overexpression of wildtype *hdac1* in the ERGs and the global pharmacological inhibition of Histone acetyltransferases (HAT) in the absence of a lesion. I found that the expression of wildtype *hdac1* in the non-lesioned larvae could stimulate ERG proliferation and that treatment with a HAT inhibitor led to an increase in neurogenesis. This suggests that a decrease in acetylation levels in the ERGs triggers them to leave their quiescent state and start producing neurons.

In conclusion, I demonstrate for the first time that Hdac1 activity within the ERGs is necessary for successful regeneration of neurons after spinal cord injury. I also show that a decrease in acetylation could be sufficient to alter the activation status of ERGs in the non-lesioned spinal cord. The new transgenic lines will be used to further investigate the interactions between extrinsic signals and regenerative neurogenesis. These insights into ERG activation in zebrafish may inform therapeutic strategies for mammalian spinal cord injury.

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Chapter 1 General Introduction

1.1 Tissue Repair of the Central Nervous System

When the mammalian central nervous system is injured the damage cannot be repaired fully (Wilkins, 1964). This leads to permanent functional deficits e.g. paralysis after spinal cord injury. Repair of the central nervous system (CNS) faces more difficulties than repair in other tissues for several reasons. There is a variety of cell subtypes and each have multiple steps involved in their differentiation. The CNS also has spatial complexity as specific cell types need to be located in certain areas and connect to specific targets. The types of injury that can occur are heterogenous. These require different repair processes. A spinal cord lesion or stroke will induce cell death in a number of cell types (David, Zarruk and Ghasemlou, 2012). In comparison neurodegeneration will involve only specific cell populations dying, such as dopaminergic neurons in Parkinson's disease (van den Berge, van Strien and Hol, 2013).

Progenitors in the CNS can contribute to the repair by replacing the lost neurons (Gage and Temple, 2013). There are populations of endogenous progenitors within the CNS of many species (Alunni and Bally-Cuif, 2016). In development, the progenitors divide and differentiate into the different neuron types to form the CNS. In adulthood they consist of two populations; active or quiescent. Active progenitors will continue to produce new neurons into adulthood whereas quiescent do not. All species have active and quiescent populations but the extent and efficacy of how these cells can participate in repair is different.

1.1.1 Comparison of the properties of stem cells between tissues

Stem cells also exist in other tissues to restore from and function after damage. In the gut the intestinal stem cells are the columnar cells located near the base of the intestinal crypt (Barker *et al.*, 2007; Tian *et al.*, 2011). Muscle precursor satellite cells are the source in skeletal muscle (Wang and Rudnicki, 2012). The bone marrow has two populations of stem cells; haemopoietic stem cells which can generate all types of blood cells (Wilson *et al.*, 2008) and stromal stem cells which can generate the bone, cartilage and fat cells (Krebsbach *et al.*, 1999). After chronic injury or bile injury in the liver, ductal progenitor cells generate the new hepatocytes (Español–Suñer *et al.*, 2012). The kidney contains progenitors in the parietal epithelium of the Bowman capsule (Diep *et al.*, 2011; Romagnani, Lasagni and Remuzzi, 2013). Stem cells regardless of source have three basic properties; they are capable of self renewal,

are unspecialised and give rise to specialised cells. Across the different tissues progenitors show similarities, such as modulation of differentiation by extrinsic signals such as Hedgehog (Reimer *et al.*, 2009; Ochoa *et al.*, 2010) and Wnt (Boulter *et al.*, 2012; Huch *et al.*, 2013; Briona *et al.*, 2015). Specific stem cell niche conditions are required for them to contribute to repair correctly (Mendelson and Frenette, 2014) including the recruitment of components of the immune system (Pull *et al.*, 2005; Ziv *et al.*, 2006; Tidball and Villalta, 2010; Anders, 2014) . Many tissues also show age related decline of regeneration (Schmitt and Cantley, 2008; Carosio *et al.*, 2011), suggesting that some properties of the stem cells have been altered with time. Across and within tissues, stem cells can display differences in activity, for example the gut stem cells renew the gut epithelium every 5 days (Tian *et al.*, 2011) whereas other populations are quiescent e.g. spinal cord progenitors (reviewed below). Future work looking at the differences and commonalities of stem cell properties between different organs and species may provide information on the biology of regeneration that can be harnessed therapeutically.

1.1.2 Regenerative neurogenesis in adult mammals

For a long time, the mammalian CNS was considered to be entirely post-mitotic past development, following the view of the pioneering scientist Santiago Ramón y Cajal in 1928 '*Everything may die, nothing is regenerated*' (Ramon y Cajal, 1928). This seemed to explain the lack of endogenous repair of the mammalian CNS following injury or disease. The discovery of neural stem cells in the adult mammalian brain (Kaplan and Hinds, 1977; Richards, Kilpatrick and Bartlett, 1992) led to work that changed this view. The mammalian CNS, today, is instead viewed as an organ capable of limited self-repair and regeneration. In the rodent brain active progenitors exist in certain locations such as the subventricular zone (SVZ) of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo, 2002) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. The same zones exist in the human brain and show constitutive neurogenesis, though the extent has recently been disputed. The dentate gyrus from healthy human brains ranging from 17-79 years old were examined and the presence of hippocampal progenitor cells were detected with immunohistochemistry. The numbers of these cells were found to remain constant with age, as well as the numbers of neurons (Boldrini *et al.*, 2018). Conversely, a different study using much younger brains observed very little neurogenesis after 6 months of age. In the adult brains they found that neurogenesis in the dentate gyrus was an extremely rare event to a point of almost not occurring (Sorrells *et al.*, 2018).

After injury, such as stroke or selective ablation, regenerative neurogenesis from these active progenitors has been observed in the rodent brain (Nakatomi *et al.*, 2002; Chen, Magavi and Macklis, 2004; Ohab *et al.*, 2006). After stroke, that generated cortex damage, GFAP-positive progenitors from the SVZ migrated to the injury site and contributed to neurogenesis there. When signalling between endothelial cells and the progenitors was altered the numbers of neurons (DCX^+) in the injury area was increased which correlated with improved behavioural performance on the whisker-guided forelimb extension task (Ohab *et al.*, 2006). Ischemic injury in the hippocampus resulted in neurogenesis ($BrdU^+$/ $NeuN^+$) from periventricular progenitors that migrated to the injury site. The numbers of new neurons detected late after injury (day 28) could be increased after treatment with growth factors and these treated mice showed improved performance on the water maze (Nakatomi *et al.*, 2002). Selective ablation of corticospinal motor neurons and the anterior neocortex by chromophore photoactivation resulted in the production of new born neurons ($BrdU^+$/$NeuN^+$) (Magavi, Leavitt and Macklis, 2000; Chen, Magavi and Macklis, 2004). A subset of these new neurons displayed an ability to reconnect into their respective networks. The exact location or identity of the progenitors responsible for this neurogenesis was not shown. Altogether, these studies highlight the ability of active endogenous progenitors in the mammalian CNS to respond to injury. They show that this response is biologically relevant as enhancing the recruitment of progenitors and subsequent neurogenesis correlated with improved functional recovery.

The neurogenesis that these active progenitors perform, however, would not be sufficient to repair all types of injury. It is an inefficient process as the animals never fully recover to uninjured functional levels. Furthermore, these progenitor cells are only located in certain areas of the CNS. Other regions of the CNS, e.g. the spinal cord, are located too far from them. These distant areas need the quiescent progenitors that are located nearby to be activated. Quiescent progenitors exist in the retina, called Müller glia, and the spinal cord. Mammalian quiescent progenitors often do not respond to injury in the same manner as the progenitors in regenerating species. For example, the neurogenic transcription factor ascl1 which is upregulated in injured zebrafish retina is not upregulated in mammals. Regeneration can be improved in the mammalian system by artificially upregulating this transcription factor (Jorstad *et al.*, 2017). This demonstrates that mammalian quiescent progenitors can be made to be more repair-like.

The mammalian spinal cord contains neural progenitors that are still present into adulthood (Meletis *et al.*, 2008). They are located in the region lining the central canal of the spinal cord and display ependymal cell characteristics such as cell markers and morphology. They show neurogenic potential when cultured *in vitro*. They are capable of self renewal and do not generate cells that leave the ependymal layer under physiological conditions, indicating they are quiescent. After injury, however, these progenitors are activated and migrate from the ependymal zone. They proliferate and differentiate but fail to generate neurons. They instead make other cells of the CNS such as oligodendrocytes and astrocytes (Barnabé-Heider *et al.*, 2010). Interestingly, the mammalian spinal progenitors do have the capability of generating neurons when placed into a suitable environment (Shihabuddin *et al.*, 2000). The reduced neurogenic potential of adult neural progenitors in the spinal cord could be due to their inability to express neurogenic transcription factors and notch signalling. After the overexpression of neurogenic transcription factor Ngn2 or a dominant negative notch ligand *in vitro* the progenitor cells increased their ability to generate neurons (Shin-ichi Yamamoto *et al.*, 2001). Notch signalling was detected *in vivo,* as well, indicating it may be one of the factors that limit the production of new neurons after spinal cord injury.

At the moment there is no therapeutic strategy that leads to successful repair and functional recovery for humans after spinal cord injury. Functional recovery in mammals after injury may be improved by stimulating the progenitors to be more neurogenic during repair. There are a number of possible approaches that one could take to discover mechanisms that are capable of altering the progenitor potential. One is to study a vertebrate that can repair its spinal cord, for example the zebrafish. Studying the mechanisms by which successful repair occurs can be an informative way to understand which pathways boost or limit progenitor potential to contribute to repair.

1.2 Zebrafish as a model organism for regeneration

Danio rerio (zebrafish) are non-mammalian vertebrates that are popular models for scientific research. They have many of the same genes as humans with ~70% of protein coding genes in humans having at least one zebrafish homologue and 82% of disease causing genes in humans having a zebrafish equivalent (Howe *et al.*, 2013). Like other fishes and amphibians, they have regenerative capabilities that are much higher than those of mammals. They are able to regenerate many organs after injury

including the tail fin (Wehner *et al.*, 2014), heart (Poss, Wilson and Keating, 2002; Zhao *et al.*, 2014; Karra *et al.*, 2015) and pancreas (Wang *et al.*, 2015; Schmitner, Kohno and Meyer, 2017).

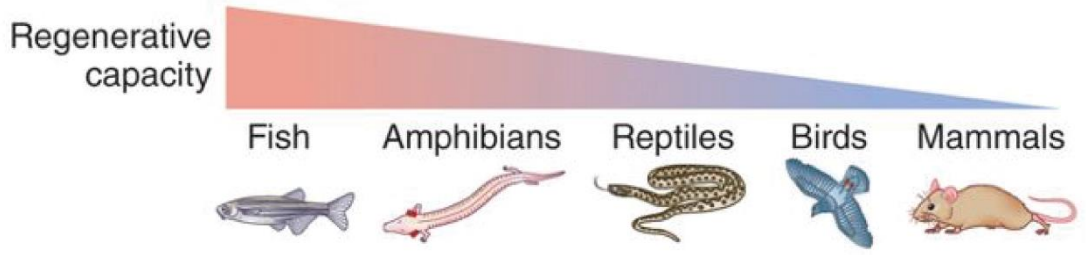


Figure 1-1: Comparison of Regeneration capacity across species. From (Forbes and Rosenthal, 2014).

1.2.1 Zebrafish and nervous system regeneration

The regenerative capability of the zebrafish also extends to the CNS. A range of injury types within the brain and spinal cord in zebrafish lead to several repair processes that then lead to functional recovery, for example, stab lesions to the dorsal telencephalon (Kroehne *et al.*, 2011; Kyritsis *et al.*, 2012), selective neuronal ablation in the brain and spinal cord (Godoy, 2015; McPherson *et al.*, 2016; Ohnmacht *et al.*, 2016; Caldwell *et al.*, 2018) and spinal cord transections (Becker *et al.*, 1997). The zebrafish spinal cord can repair in two manners; the existing neurons can regrow their axons and reconnect with their targets (axonal regeneration) and progenitors within the CNS can proliferate and differentiate into the lost neuronal cell types (regenerative neurogenesis).

Axonal regeneration is necessary for functional recovery after spinal cord injury. If axons are prevented from growing across the lesion site in adult zebrafish by a physical barrier, recovery of full swimming function is not achieved (Becker *et al.*, 1997). When re-lesioned the behavioural recovery is abolished again (Kuscha, Barreiro-Iglesias, *et al.*, 2012). The same occurs in larval zebrafish, where axonal regeneration correlated with distance swum after injury (Wehner *et al.*, 2017). Many signals have been discovered to be necessary for the axons to grow across the lesion site. It has been proposed that the axons needed support from glial cells; termed a glial bridge, to be able to grow across the lesion site (Goldshmit *et al.*, 2012). These glia cells secrete extracellular molecules such as connective tissue growth factor (ctgfa) (Mokalled *et al.*, 2016) and fibroblast growth factor (fgf) (Goldshmit *et al.*, 2012)

that directs the glial bridge formation. These in turn then promote axonal regeneration across the lesion site. Recent evidence from our group indicates that axons in fact do not need a glial bridge to cross and can cross the lesion site without glial cells being present. A Wnt-dependent factor extracellular matrix protein Collagen XII has been found to be necessary for and promote axon growth across the lesion site (Wehner *et al.*, 2017). This result suggests the non-neural lesion environment is what is important for axonal regeneration and through conditioning of this extracellular matrix recovery could be improved further.

Regenerative neurogenesis in zebrafish CNS is performed by progenitors that exist in the same locations as in the mammalian CNS (Reimer *et al.*, 2008; Kroehne *et al.*, 2011). They have ependymal radial glial characteristics and have been called ependymo-radial glial (ERG). They express a range of genes such as radial glia markers; sox2, vimentin, brain lipid binding protein (BLBP), glutamate aspartate transporter (GLAST), glial fibrillary acidic protein (GFAP) and ependymal markers Foxj1 and s100β (Kroehne *et al.*, 2011; Hui, Nag and Ghosh, 2015; Ribeiro *et al.*, 2017). In the brain, genetic lineage tracing found that the notch target gene *her4.1* also labelled the ERGs (Kroehne *et al.*, 2011). Transgenic lines using these markers can be used to label the cells *in vivo*. In the adult spinal cord, the ERGs occupy set domains that mirror the domains set up during development. They are defined by the expression of different transcription factors e.g. nkx6.1, shh, pax6, olig2, dbx1 (Reimer *et al.*, 2009; Briona and Dorsky, 2014). The overlap and/or exclusion of the expression of these transcription factors leads to specific ERG populations that reside in specific locations along the dorsal-ventral axis. These ERG populations give rise to different neuronal and glial cells; the pMN (yellow domain in Figure1.2) produces the motor neurons and the oligodendrocytes.

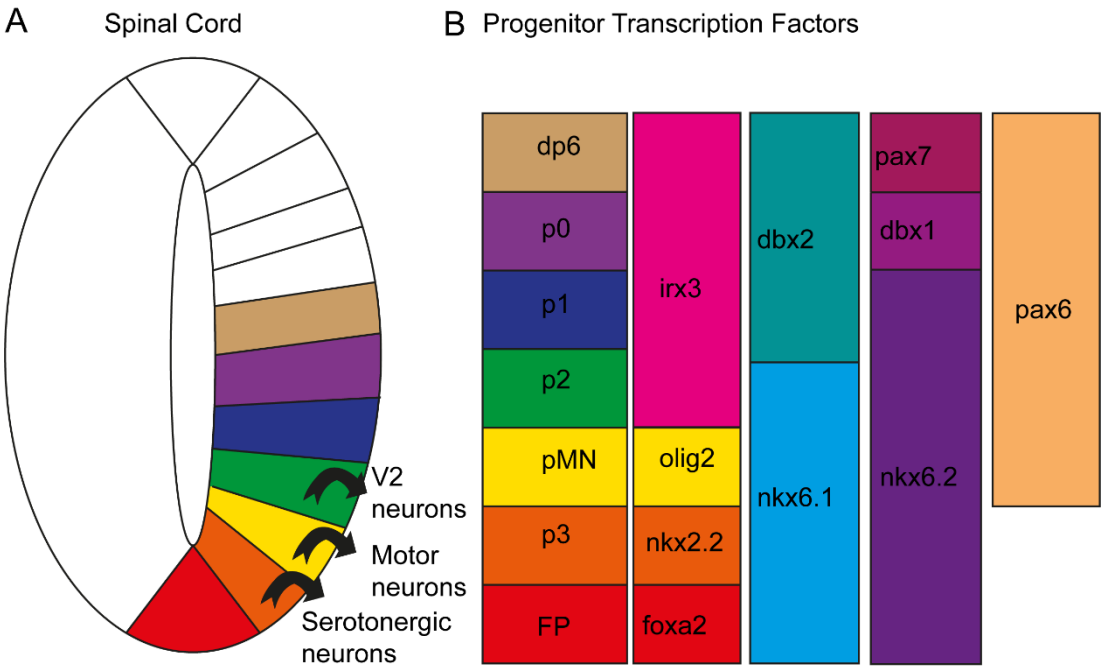


Figure 1-2: Distinct progenitor domains in the spinal cord give rise to distinct neuronal populations and are set up by the expression of transcription factors. **A**: Schematic of a spinal cord cross section depicting the position of some of the ERG domains. **B**: Table of the expression of the transcription factors depicting the domains in which they are expressed.

After a spinal cord injury the ERGs proliferate as shown by PCNA immunohistochemistry with a peak at 2 weeks post injury (Reimer *et al.*, 2008; Hui, Nag and Ghosh, 2015). The same populations of ERGs generate the lost neuronal subtypes in adults as they do in development. After injury new born motor neurons labelled with Hb9- antibody can be found to co-label with *olig2*:GFP, as the relative stability of GFP acts as a lineage tracer, showing they arise from the pMN-like domain (Reimer *et al.*, 2008). New born *vsx1*:GFP interneurons are regenerated from the p2-like domain which are nkx6.1^{+}/pax6^{+}/olig2^{-} (Kuscha, Frazer, *et al.*, 2012). Serotoninergic interneurons regenerate from the p3-like domain (Kuscha, Barreiro-Iglesias, *et al.*, 2012). Some progenitors are not induced to produce their neuronal subtypes after lesion. Parvalbumin 7-positive interneurons are very rarely (2%) new born after a lesion in the adult spinal cord (Kuscha, Frazer, *et al.*, 2012). The generation of pax2-positive neurons is not enhanced in the larval spinal cord after lesion (Ohnmacht *et al.*, 2016). Therefore, progenitor domains can differ in their regenerative potential.

The exact function that regenerative neurogenesis has in functional recovery after spinal cord injury has not been as extensively demonstrated as axonal regeneration. When the numbers of new born motor neurons was increased by 61% after the

inhibition of notch signalling (Dias *et al.*, 2012) or decreased by 50% after inhibition of hedgehog signalling (Reimer *et al.*, 2009) no significant difference was observed in swimming behaviour after injury in the adult zebrafish spinal cord. There are hundreds of new motor neurons made within the two weeks after injury but labelling has found that many do not become fully mature. The number of ChAT positive mature motor neurons at 6-8 weeks post-lesion is much lower than the numbers of immature *hb9*:GFP motor neurons observed at 2 weeks post-lesion (Reimer *et al.*, 2008). The presence of mature neurons may be what is necessary for function to recover and a sufficient number are replaced even after reductions in the generation of the immature population. Improved performance, also, may be harder to observe in zebrafish which have a high repair capacity. In mammals where neurogenesis is minimal, an increase in the numbers of neurons generated could lead to observable functional improvements. In support of this, increasing neurogenesis($BrdU^+/DCX^+$, $BrdU^+/Hu^+$, $BrdU^+/NeuN^+$) by endogenous progenitors after transplantation of dendritic cells or adult neural progenitor cells alongside vaccination against CNS antigens in lesion site after spinal cord injury correlated with improved performance in behavioural assays such as Basso-Beattie-Bresnahan (BBB) (Mikami *et al.*, 2004; Ziv *et al.*, 2006). These studies, however, do not rule out axonal regeneration being the cause of the improved functional recovery. Fgf2 treatment after injury in the mammalian spinal cord led to an increased amount of neurogenesis ($BrdU^+/DCX^+$) that correlated with better functional outcomes; grid walking and modified BBB. The authors claim that since not many axons have crossed the lesion site when testing was carried out (under 7 weeks post lesion) the behavioural change could not be fully explained by axon regeneration (Goldshmit *et al.*, 2014). Therefore, the discovery of signalling pathways that are involved in successful regeneration could see beneficial effects in the mammalian system.

1.3 Extrinsic signals involved in regenerative neurogenesis

The ERGs receive many extrinsic signals that are released by other cells and found in the lesion site. Though the ERGs in the spinal cord are in a quiescent state normally they have been found to express a vast array of receptors (Reimer *et al.*, 2009, 2013; Barreiro-Iglesias *et al.*, 2015). They are not unobservant to numerous different possible signals that are released after an injury.

1.3.1 Hedgehog

Hedgehog (HH) is a morphogen that is released in the spinal cord by cells in the floor plate. It is a positive regulator of neurogenesis, ectopic expression can induce motor

neuron differentiation (Ericson *et al.*, 1996; Chandrasekhar *et al.*, 1998) while preventing the signal reduces differentiation (Chiang *et al.*, 1996; England *et al.*, 2011). The dorsal most region of the spinal cord is not dependent on HH (Pierani *et al.*, 1999). Its secretion creates a ventral-dorsal gradient and changes in concentration of HH generates the different classes of progenitors and their subsequent neuronal populations (Ericson, Briscoe, *et al.*, 1997). ERGs continue to express HH receptors into adulthood and it plays a role in adult neurogenesis in the mammalian brain (Ahn and Joyner, 2005; Palma *et al.*, 2005; Balordi and Fishell, 2007). Hippocampal progenitor proliferation was increased when HH was overexpressed whereas proliferation was reduced after antagonist treatment (Lai *et al.*, 2003) or conditional knockout of signalling (Machold *et al.*, 2003).

As well as being important in development of the CNS, it has been found to have a positive role in the regeneration of many tissues including neural such as the retina (Spence *et al.*, 2004). It is upregulated in the mammalian and in the zebrafish spinal cord after injury (Chen, Leong and Schachner, 2005; Reimer *et al.*, 2009). After an injury the pMN-like domain ERGs increase the expression of downstream pathway genes, *smoothened* and *patched1*. Hedgehog signalling has been found to have a positive role in motor neuron (Reimer *et al.*, 2009) and serotonergic interneuron (Kuscha, Barreiro-Iglesias, *et al.*, 2012) regeneration as treatment with the pharmacological antagonist cyclopamine reduced the numbers of the new born neurons by 50% and 23% respectively. Treatment with a pharmacological agonist in the nonlesioned spinal cord did not elicit a response from the progenitors and whether this agonist boosted motor neuron numbers in regeneration was not tested.

1.3.2 Notch

Notch signalling is a cell-cell communication process that is important for regulating the development of neurons. Neurons express notch ligands (delta/jagged) that bind to notch receptor on progenitors. This leads to activation of intracellular signalling pathways within the progenitor that controls its proliferative activity and prevents neuronal differentiation. When notch signalling is reduced in development there is an increase in the numbers of motor neurons generated (Itoh *et al.*, 2003; Kim *et al.*, 2008). As the zebrafish spinal cord develops the expression of different notch pathway is reduced by 3dpf which correlates to the end of embryogenesis (Kim *et al.*, 2008). In the adult zebrafish brain notch activity controls their quiescence. The inhibition of notch with DAPT treatment was sufficient to increase neurogenesis in the telencephalon (Chapouton *et al.*, 2010, 2011).

Notch pathway is upregulated in the spinal cord after a lesion in both mammals and zebrafish. The expression of many elements of the pathway are upregulated including notch target genes, receptors and ligands (Shin-ichi Yamamoto *et al.*, 2001; Dias *et al.*, 2012). Different zones of the zebrafish spinal cord showed some region specific expression of the notch pathway e.g. *her4.1* was found in the ventral zone while *her9 was* present in the dorsal zone (Dias *et al.*, 2012). Notch is thought to be a cause for the reduced regenerative capability of the mammalian spinal cord. When notch is inhibited in ERGs *in vitro* , the mammalian progenitors increased their neurogenic capability (Shin-ichi Yamamoto *et al.*, 2001). This was observed *in vivo* in adult zebrafish. When notch signalling is boosted, using a heatshock promotor to overexpress the active intracellular domain of notch1a, there was a significant reduction in the numbers of new born motor neurons generated after a lesion. Inhibition of notch, with the pharmacological inhibitor DAPT, resulted in an increase in the numbers of new born motor neurons after a lesion. The changes in motor neuron numbers in both manipulations were due to changes in ERG lesion induced proliferation (Dias *et al.*, 2012). This points to Notch playing a negative role in controlling the regeneration of motor neurons. DAPT treatment in the unlesioned spinal cord was not sufficient to lead to motor neuron generation or progenitor proliferation, unlike what had been observed in the telencephalon.

1.3.3 Fibroblast growth factor

Growth factors are part of a family of extracellular proteins that promote growth and bind to ligand specific tyrosine kinase receptors. These lead to intracellular signalling via phosphorylation cascades. FGF treatment during development in zebrafish increased neurogenesis of *islet1*:GFP^{+} motor neurons and *in vitro* increased cell proliferation of PC12 cell line which model neuronal lineages (Goldshmit *et al.*, 2018). FGF has been found to regulate neurogenesis in the adult mammalian hippocampus. Fgf2 infusion increased the number of new born neurons (Rai, Hattiangady and Shetty, 2007) while conditional knockout of its receptor, fgfr1, on the ERGs decreased their proliferation and the numbers of new neurons made (Zhao *et al.*, 2007).

The FGF signalling pathway (ligands, receptors and downstream genes) is upregulated in the mammalian and zebrafish spinal cord after a lesion (Reimer *et al.*, 2009; Goldshmit *et al.*, 2012, 2014). FGF signalling has been shown to positively modulate neuroregeneration. In adult zebrafish, inhibition of FGF with the pharmacological inhibitor SU5402 or the expression of a dominant negative receptor fgfr1, decreased the lesion induced proliferation of ERGs (Goldshmit *et al.*, 2012) and

neurogenesis ($NeuN^+$) (Goldshmit *et al.*, 2018). Increasing the levels of FGF, with spry4 $^{-/-}$ knockout animals or injection of ligand fgf8, increased the ERG proliferation (Goldshmit *et al.*, 2012) and neurogenesis ($NeuN^+$) (Goldshmit *et al.*, 2018). Fgf3 treatment after lesion in adult zebrafish spinal cord increased neurogenesis of *islet1*:GFP^+ neurons but had no effect on other neuronal types such as *vsx1*:GFP interneurons. The application of a different FGF ligand Fgf8 had no effect on *islet1*:GFP^+ positive neurons after injury. These results suggest that distinct FGF ligands mediate different responses from different ERG populations. FGF also has a role in the mammalian spinal cord after injury as fgf2 treatment led to an increase in numbers of newborn neurons after a lesion in the mouse spinal cord (Goldshmit *et al.*, 2014). Increasing FGF signalling in the intact adult zebrafish spinal cord with spry4 $^{-/-}$ mutants had no effect on neurogenesis (Goldshmit *et al.*, 2018).

1.3.4 Retinoic Acid

RA has been found to have a role in neural patterning (Wilson *et al.*, 2004) in development. In the chicken spinal cord, RA treatment increases motor neuron generation and increases the proliferation of ventral progenitors (Sockanathan and Jessell, 1998). Treatment of RA in zebrafish embryos also increases the number of motor neurons generated during development (Ryu *et al.*, 2015) and biases the proliferating progenitors towards motor neuron fates at the expense of GABAergic interneurons (Kong *et al.*, 2018). It is necessary for adult neurogenesis in mammals (Jacobs *et al.*, 2006) and is used extensively *in vitro* to direct neuronal differentiation of stem cells (Tonge and Andrews, 2010; Tan *et al.*, 2015). The RA signalling pathway, such as receptors (*rarab*, *rxrga*) and downstream genes (*crabp2a*, *cyp2ba*), is upregulated in the adult zebrafish spinal cord after a lesion (Reimer *et al.*, 2009). *Raldh2* is increased in mammalian spinal cord after injury in NG2+ cells (Mey *et al.*, 2005; Kern *et al.*, 2007). No studies so far have investigated the functional role of RA in neuroregeneration in the spinal cord.

1.3.5 Wnt

In spinal cord development, a component of the canonical Wnt signalling pathway, β-catenin controls the size of the neural precursor population (Zechner *et al.*, 2003).Wnt signalling is present in the neurogenic regions of the adult mammalian brain (Lie *et al.*, 2005). In the hippocampus, increasing Wnt signalling was able to promote hippocampal ERG proliferation (Mao *et al.*, 2009) and neurogenesis (Lie *et al.*, 2005). Expression of a dominant negative Wnt reduced hippocampal neurogenesis (Lie *et al.*, 2005). Wnt-induced neurogenesis was found to be mediated

through the activation of the NeuroD1 transcription factor (Kuwabara *et al.*, 2009). In the SVZ, increasing Wnt signalling by the overexpression of Wnts or the stabilisation of β-catenin increased proliferation and differentiation of ERGs *in vitro* (Yu *et al.*, 2006) and *in vivo* (Adachi *et al.*, 2007).

Wnt signalling is upregulated after spinal cord injury in mice (González-Fernández *et al.*, 2014). Wnt function has been found to be necessary for the regeneration of xenopus spinal cord (Lin, Chen and Slack, 2012) and locomotor recovery in adult zebrafish after spinal cord injury (Strand *et al.*, 2016). Wnt inhibition, with the pharmacological inhibitor IWR or the expression of a Wnt pathway inhibitor Dkk1, during larval zebrafish regeneration, led to a reduction in the numbers of new born neurons ($BrdU^+/HuC^+$). Wnt inhibition did not affect ERG lesion induced proliferation (Briona *et al.*, 2015). In contrast, research from our group found that there was very little activity of the Wnt pathway in the spinal cord ERGs after a lesion in larval zebrafish and when Wnt was inhibited specifically in these cells no change was observed in locomotor recovery. (Wehner *et al.*, 2017).

1.3.6 Monoaminergic Neurotransmitters

Neurotransmitters, though known primarily for their roles in neuron communication at the synapse, have been found to have a role in neurogenesis (Berg *et al.*, 2013). The monoaminergic neurotransmitter dopamine regulates neurogenesis in the adult salamander midbrain. Dopamine receptor antagonism, with haloperidol, was sufficient to cause the quiescent ERGs located there to become activated and produce neurons (Berg *et al.*, 2011). In the adult mammalian brain, reductions of dopamine levels reduced ERG proliferation in the SVZ (Baker, Baker and Hagg, 2004) while treatment with agonists increased proliferation (Höglinger *et al.*, 2004). Treatment with a dopamine D3 receptor agonist increased neurogenesis in the SVZ and neostriatum (Van Kampen, Hagg and Robertson, 2004). In the zebrafish spinal cord, dopamine has a positive role in both motor neuron development and regeneration. In development dopamine promotes motor neuron generation at the expense of the v2 interneurons (Reimer *et al.*, 2013). When descending dopaminergic axons from the brain were ablated in adult zebrafish, with 6-OHDA, the number of new born motor neurons after a spinal cord lesion was reduced. This specifically occurred in the rostral side of the spinal cord. Increasing dopamine signalling after a lesion using the pharmacological agonist R(-)-propylnorapomorphine (NPA) increased the numbers of new born motor neurons in the caudal side of the adult zebrafish spinal cord (Reimer *et al.*, 2013). Another dopamine agonist pergolide, increased the number of new born

motor neurons after a lesion in the larval zebrafish spinal cord (Ohnmacht *et al.*, 2016). The ERGs upregulate the expression the dopamine receptor 4a after a lesion. Signalling through Drd4a acts through cAMP/PKA to modulate the hedgehog pathway to influence motor neuron regeneration (Reimer *et al.*, 2013).

Another monoamine neurotransmitter, serotonin was found to have a similar effect as dopamine, with a positive role on motor neuron development and regeneration in the zebrafish spinal cord. Serotonin signalling promotes motor neuron generation while the numbers of interneurons remained unchanged. When the serotonergic descending axons were ablated with 5,7-DHT after a lesion, motor neuron regeneration was reduced in the rostral side of the adult zebrafish spinal cord (Barreiro-Iglesias *et al.*, 2015). Injections of serotonin increased the numbers of new born motor neurons after a lesion in the caudal side of the adult zebrafish spinal cord by increasing the proliferation of the ERGs. Interestingly, serotonin levels do not have a role in the regeneration of local serotonin interneurons as their numbers were not affected by the manipulations above, suggesting that it was specifically the motor neurons that were regulated by this mechanism. The mechanism appears to work independently to dopamine and not via cAMP/PKA signalling.

These signals all share a common property that they do not have any effect in the unlesioned spinal cord. The ERGs become somehow sensitive to the signals only after an unknown injury mechanism. The immune system could be such an upstream mechanism. Cells of the immune system respond quickly to an injury in the larval zebrafish spinal cord; neutrophils are recruited to the lesion site within 2 hours and macrophages are recruited by 24 hours (Tsarouchas *et al.*, 2018). They are therefore in place to signal to the ERGs to sensitise them to then additional signals that then arrive.

1.3.7 Immune system

Immune signalling has been found previously to have a role in the regeneration of the zebrafish CNS. In the adult telencephalon, boosting the immune system with zymosan A, a ligand on the surface of fungi which induces inflammation, had a beneficial effect on regenerative neurogenesis. Immunosuppression with the corticosteroid dexamethasone reduced the lesion induced neurogenesis (Kyritsis *et al.*, 2012). The chemokine leukotriene 4 (LTC4) could directly interact with the ERGs in the telencephalon ventricle which expressed its receptor CysLT1. LTC4 signalling and the zymosan induced immune response was found to be sufficient to elicit a

proliferation response from the progenitors and stimulate neurogenesis in the brain even in the absence of a lesion. Another chemokine receptor Cxcr5 was upregulated in the telencephalon ERGs after a stab lesion to the adult telencephalon (Kizil, Dudczig, *et al.*, 2012). Heatshock overexpression of either wildtype or dominant negative Cxcr5 demonstrated that this chemokine had a positive role in proliferation and neurogenesis after a lesion. Unlike LTC4, however, in the absence of a lesion Cxcr5 overexpression was not sufficient to stimulate neurogenesis. These results support the significant role the immune response has in CNS regeneration and show that some factors of the immune system have different roles in the repair process.

As mentioned earlier the progenitors in the zebrafish brain, though similar in many respects to the ones found in the spinal cord, do have one significant difference. The progenitors in the adult brain are part of the active population and contribute to continuous neurogenesis. The ERGs in the spinal cord, on the other hand, are completely quiescent after development has finished. There is evidence that the neurogenesis caused by the inflammation signals can induce the expression of an injury specific transcription factor Gata3 (Kizil, Kyritsis, *et al.*, 2012). The neurogenesis in the brain, that is stimulated by the immune system, can therefore be claimed to be different to the constitutive neurogenesis that is ongoing in this region (Kyritsis *et al.*, 2012). However, due to their active neurogenesis the brain progenitors may be in a more plastic state than the ERGs in the spinal cord, which makes them more amenable to respond to the inflammation signals.

Previous work in our group has shown the immune system has a beneficial role in spinal cord regeneration. When the immune system is supressed by dexamethasone treatment motor neuron regeneration in larval zebrafish is reduced (Ohnmacht *et al.*, 2016). This reduction also occurs when some of the cellular elements of the immune system are removed. In the irf8$^{-/-}$ mutant there is a delay in development of macrophages and microglia until timepoints after the larval lesion is performed (over 5dpf) (Shiau *et al.*, 2015). The irf8$^{-/-}$ mutants do not regenerate as well as wildtype siblings in terms of both axonal regeneration (Tsarouchas *et al.*, 2018) and regenerative neurogenesis (unpublished observations). Therefore, an appropriate immune response is necessary for successful regenerative neurogenesis to occur in both the zebrafish brain and spinal cord.

1.4 Intrinsic mechanisms

The activation of many of the extrinsic signals detailed above does not stimulate the ERGs in the unlesioned spinal cord, suggesting that progenitor cell intrinsic programs also play essential roles in neuroregeneration. The intrinsic changes may change the responsiveness of the ERGs to fate regulating extrinsic signals. Studies investigating the extrinsic signals are often studied in isolation. After an injury, however, the ERG will receive all the signals simultaneously. Studies looking at a single pathway do not inform us how the ERGs integrate the vast array of signals to lead to successful regeneration. The downstream mechanisms that these different signalling pathways trigger also remain unclear. It is unlikely that the expression of one single gene is responsible for successful regeneration but that a mechanism that can control the expression of a gene programme is needed. The differential expression of transcription factors or changes in epigenetic modifications are two intrinsic mechanisms that control the expression of many genes.

1.4.1 Transcription factors

Transcription factors bind to certain sequences of DNA to regulate the expression of other genes. The expression of many of the developmental transcription factors have been found to increase after injury in the adult zebrafish spinal cord (Reimer *et al.*, 2008; Hui *et al.*, 2014). Pax6 is a homeodomain protein which is repressed by HH (Briscoe *et al.*, 2000). Pax 6^{+} progenitors in the spinal cord give rise to V1, V2 interneurons and motor neurons. Without Pax6 there is a dorsal-to-ventral transformation of the progenitor identity (Ericson, Rashbass, *et al.*, 1997; Takahashi and Osumi, 2002). In the developing mammalian cortex Pax6 levels in the progenitors control the balance between self-renewal and neurogenesis (Sansom *et al.*, 2009). In adult mammalian olfactory bulb (Hack *et al.*, 2005) and hippocampus (Klempin, Marr and Peterson, 2012) Pax6 expression in progenitors promotes them towards a neuronal fate and is sufficient to instruct neurodifferentiation (Kohwi *et al.*, 2005). In the mammalian spinal cord Pax6 increases after injury around the central canal and in the parenchyma (S Yamamoto *et al.*, 2001). This injury induced increase was also observed in the adult zebrafish spinal cord (Reimer *et al.*, 2008; Hui *et al.*, 2014).

Olig2 is a transcription factor that is specific to the pMN domain that first produces motor neurons and then switches to producing oligodendrocytes. Olig2 expression is induced by HH signalling (Lu *et al.*, 2000). In developmental neurogenesis, Olig2 is necessary and sufficient for the generation of motor neurons and oligodendrocytes in mammals (Takebayashi *et al.*, 2002) and zebrafish (Park *et al.*, 2002) . In the adult

mammalian SVZ, Olig2 is expressed in progenitors but exerted the opposite effects to Pax6. When Olig2 is downregulated, the progenitors can proceed to neurogenic lineages (Hack *et al.*, 2004, 2005; Marshall, Novitch and Goldman, 2005). Olig2 positively regulates adult neurogenesis in the hippocampus, however, blocking its action decreases net neurogenesis there and expands the astrocyte lineage (Klempin, Marr and Peterson, 2012). After brain injury Olig2 expression was found to be upregulated and repressors neurogenesis. Antagonism of Olig2 function led to an increase in the number of neurons after stab injury to the cortex (Buffo *et al.*, 2005) and stroke injury to the striatum (Kronenberg *et al.*, 2010). The same studies found that overexpression of Pax6 was able to increase neurogenesis after injury. Olig2 expressing cells are the progenitors that generate new motor neurons after injury in the zebrafish spinal cord (Reimer *et al.*, 2008; Ohnmacht *et al.*, 2016). No functional studies have been performed yet to demonstrate the functional role Pax6 or Olig2 have in neuroregeneration in the spinal cord.

Two members of the Sox family of transcription factors have been identified as having roles in spinal cord regeneration; sox2 and sox11b (Guo *et al.*, 2011; Ogai *et al.*, 2014). These transcription factors are characterised by the high mobility group (HMG)- box sequence motif through which they bind to DNA. Sox2 has roles in mammalian CNS development (Zappone *et al.*, 2000; Ferri *et al.*, 2004) and in the regeneration of the *Xenopus* spinal cord (Gaete *et al.*, 2012). Sox2 expression decreases as the *Xenopus* ages which the authors speculate correlates with the reduced regenerative ability of the adult. Sox2 expression increases in adult zebrafish ERGs after spinal cord injury and expression precedes markers of proliferation. When sox2 was inhibited with morpholino the number of PCNA-positive cells was reduced (Ogai *et al.*, 2014). Sox 11 is expressed in neural progenitors that are committed to neuronal differentiation (Uwanogho *et al.*, 1995; Kamachi, Uchikawa and Kondoh, 2000). In adult mammals expression of sox11 is mainly in neurogenic areas and overexpression of sox11 in adult neural stem cells *in vitro* promotes the generation of neurons (DCX^+) (Haslinger *et al.*, 2009). Expression of one of the zebrafish homologues sox11b is upregulated in optic nerve regeneration (Veldman *et al.*, 2007) and spinal cord (Guo *et al.*, 2011) in adult zebrafish. Inhibition of sox11b with morpholino during regeneration reduced proliferation in the spinal cord and reduced the distance swum at 6 weeks after injury. They do not show, however, that this is due to previous effect on proliferation or whether axon regeneration is also affected by the sox11b morpholino.

1.4.2 Epigenetic modifications

Epigenetics is the study of changes in gene expression that is caused by alterations that are not due to changes in the DNA sequence. The patterns of gene expression are regulated by altering DNA accessibility and chromatin structure. DNA can be methylated at cytosine residues by enzymes called DNA methyltransferases (DNMTs). A range of other enzymes can add on additional modifications such as oxidation and deamination. These enzymes have been found to be expressed in progenitors during development (Goto *et al.*, 1994) and have functional roles in neurogenesis (Wu *et al.*, 2010). After retinal injury in zebrafish the Mülller glia transition from a quiescent state to a progenitor cell. During this transition they upregulate DNA methylation/demethylation machinery. Treatment with a demethylase inhibitor enhanced the Müller glia programming after injury though reduced their proliferation, migration and differentiation (Powell *et al.*, 2013). During reprogramming the DNA methylation landscape changes, towards demethylation, which may lead to changes in gene expression during this time. However, some of the well-known regeneration genes did not change DNA methylation state, indicating that other mechanisms are also involved such as histone modifications.

DNA is packaged into highly ordered chromatin structures in eukaryotes by wrapping around histone proteins. The amino terminal 'histone tails' of the proteins can be post translationally modified such as methylation and acetylation. These modifications change the charge of the proteins and their interactions with the DNA. In developmental neurogenesis, histone methylation and acetylation changes at ngn1 promoter correlate with *ngn1* mRNA expression of neural progenitors in mouse cortex (Hirabayashi *et al.*, 2009). These histone modifications are proposed mechanisms the progenitors uses to maintain its self-renewal capabilities and to alter their fate choices between neurogenesis and gliogenesis (Hirabayashi *et al.*, 2009; Pereira *et al.*, 2010). Histone demethylase enzymes are expressed in the adult zebrafish fin during regeneration. One enzyme, kdm6b.1, was expressed only in the blastema and displayed the highest upregulation during regeneration. Morpholino knockdown of kdm6.1 showed it is necessary for regeneration of the caudal fin in zebrafish larvae. Kdm6b.1 was suggested to function in regeneration by regulating the expression of dlx4a, a gene that functions in fin/limb development (Stewart, Tsun and Belmonte, 2009). Another common modification of the histones is acetylation.

1.4.3 Acetylation

Acetylation levels in the cell are reversibly controlled by two classes of enzymes. Histone deacetylases (HDACs) are the class of enzymes that remove acetyl groups from lysine residues on proteins. They work in competition with Histone acetyltransferases (HATs) which add on the acetyl groups. There are 18 HDACs in humans; Class I (HDAC1,2,3,8); Class IIa (HDAC4,5,7,9); Class IIb (HDAC6 and 10), Class III (sirtuins) and Class IV (HDAC11) shown in Figure 1.3.

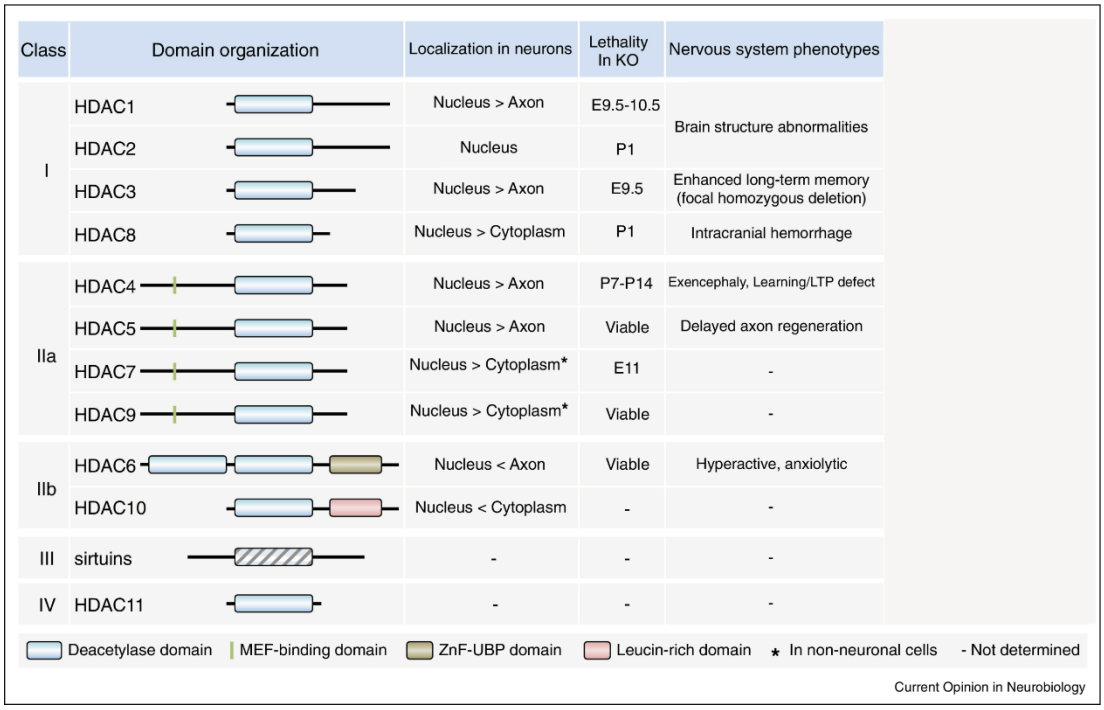


Figure 1-3: Table of the different HDAC genes. The different enzymes are separated into Classes with information on their general structural domains, localisation in neurons and phenotypes observed in mouse mutants. Adapted from (Cho and Cavalli, 2014).

HDAC1 was the first to be discovered in mammalian cells (Taunton, Hassig and Schreiber, 1996). HDACs were found to have a role in gene repression as the deacetylase action on histone proteins leads to chromatin becoming more compacted. This compaction is thought to make it harder for transcription factors to access the DNA and therefore lead to repression. Additionally, the acetyl-lysine residues in the histone tails may serve as a signal for bromodomain-containing transcriptional regulators (Winston and Allis, 1999). The removal of this signal might impede the assembly or recruitment of transcriptional activators. More evidence now suggests that the action of HDACs also leads to as many genes being activated as repressed (Zupkovitz *et al.*, 2006; Wang *et al.*, 2009; Greer *et al.*, 2015). The action of HDAC enzymes is not limited to histone proteins. They can interact with many non-

histone proteins that have lysine residues in the cell, for example, transcription factors. This modification can change the activity of that transcription factor. In hedgehog signalling the Gli transcription factors can be acetylated, and Gli1/2 become more active when deacetylated (Canettieri *et al.*, 2010; Coni *et al.*, 2013).

Class I HDACs except for HDAC8, are assembled in the cell into multi-subunit corepressor complexes. The enzymatic activity of the HDACs are enhanced by recruitment into these complexes. HDACs are part of several distinct corepressor complexes; NuRD, Sin3, CoREST, MiDAC and SMRT/NCoR (Hui Ng and Bird, 2000). These are then recruited to DNA via interaction with DNA binding factors. Nucleosome remodelling and deacetylase complex (NuRD) couples together two important enzymatic functions; ATP-dependent nucleosome remodelling by chromodomain helicase DNA binding proteins Mi2, with the deacetylase activity of HDAC1/2 (Xue *et al.*, 1998). NuRD binds to the promoters of genes involved in pluripotency (Reynolds *et al.*, 2012). Sin3-HDAC complex couples the deacetylase with a paired amphipathic helix (PAH) domain containing protein. This complex is recruited by DNA binding transcription factors such as the Mad/Max heterodimer (Hassig *et al.*, 1998), regulates STAT transcriptional activity (Icardi *et al.*, 2012) and maintains pluripotency (Saunders *et al.*, 2017). CoREST is the corepressor protein to the transcription factor REST (RE1 silencing transcription factor/neural silencing factor) (You *et al.*, 2001). HDAC-CoREST mediates the repression of genes responsible for the neuronal phenotype, such as specific sodium channel expression, in non-neuronal cells (Andrés *et al.*, 1999; Ballas *et al.*, 2005). Mitotic deacetylase complex (MiDAC) is named for its high abundance in cells arrested in mitosis (Bantscheff *et al.*, 2011). The SMRT/NCoR (silencing mediator for retinoid and thyroid receptors/ nuclear receptor corepressor) complex exclusively recruits HDAC3 (Watson *et al.*, 2016) and interacts with unliganded nuclear hormone receptors. These different complexes act as protein platforms facilitating the recruitment of HDACs to the DNA promoters bound by their interacting transcription factors. Therefore, changes in HDAC can have wide range of effects on the gene expression of a cell.

In zebrafish Hdac1 is the orthologue of HDAC1 and 2 in mammals. In mammals these two forms can compensate for each other suggesting some functional redundancy (Montgomery *et al.*, 2009). The role of this protein in regeneration can be studied in zebrafish without this compensation. Other Hdac genes have been found in zebrafish including; Hdac3 (Farooq *et al.*, 2008), Hdac4 (DeLaurier *et al.*, 2012), Hdac5 (Just *et*

al., 2011), Hdac6 (Kaluza *et al.*, 2011) and Hdac8, 9a/b, 10,11 (Huang *et al.*, 2013). Hdac1 was selected to be studied further in the process of neuroregeneration because of the evidence of the role of this HDAC form in neurogenesis during development.

1.4.4 HDAC1 in the development of the nervous system in mammals

Acetylation has a role in the fate decisions of neural progenitor cells in development (Tapias *et al.*, 2014) and is needed for correct adult neurogenesis (Merson *et al.*, 2006). HDAC1 expression can be found in cells of the CNS during development. Global HDAC1 knockout in mice are embryonic lethal before day 10.5 after gestation but show abnormalities throughout growth (Lagger *et al.*, 2002; Montgomery *et al.*, 2007). HDAC1 is expressed at higher levels in glial progenitors compared to neurons, suggesting that it is downregulated as differentiation occurs (MacDonald and Roskams, 2008; Foti *et al.*, 2013).

Different studies have looked at the role of HDAC1 in neurogenesis in mammals with conflicting results. In PNS development depletion of HDAC1/2 in neural crest cells using the Wnt1-Cre reduces the number of sensory neurons (*Isl1,Neurod*) generated (Jacob *et al.*, 2014), indicating that HDAC1/2 controls the specification of neural crest cells into peripheral glia. Depletion of HDAC1/2 in CNS progenitors using GFAP-Cre showed CNS defects and lethality (Montgomery *et al.*, 2009). Mice that lacked only one gene had no observed deficits. The brains of $HDAC1^{loxP/loxP}$; $HDAC2^{loxP/loxP}$; GFAP-Cre showed increased proliferation of progenitors at ventricle zones but reduced numbers of neurons ($Tuj1^{+}$). When cortical neuronal precursors were taken from these mice and then put through an *in vitro* differentiation assay they failed to produce neurons. The progenitors ability to generate astrocytes, however, was not changed (Montgomery *et al.*, 2009). Deletion of HDAC1/2 in oligodendrocytes lineage cells using Olig1-Cre led to complete absence of mature oligodendrocytes (*Mbp,Plp*) and precursors (*Pdgfrα, Olig2,NG2*) in the spinal cord and brain (Ye *et al.*, 2009). No deficits were observed in motor neuron (*Hb9, Isl2*) or astrocyte (*Gfap*) development. Further experiments showed that HDAC1/2 is necessary in the progenitor to supress Wnt activity to enable oligodendrocyte development. Transfection of rat foetal cortical stem cells with a dominant negative form of HDAC1 decreased their ability to generate neurons (βIII-tubulin^{+}). The expression of wildtype HDAC1 in these cells caused no changes in numbers of neurons generated (Humphrey *et al.*, 2008). HDAC inhibitor treatment or conditional depletion of HDAC1 inhibited neural differentiation of mouse embryonic stem cells (mESCs). When HDAC1 knockout mESCs where injected into

blastocysts to generate chimeric animals, the incorporation rate into neural tissues of the HDAC1 knockout cells was significantly reduced compared to wild type cells. The major target of HDAC1 in this process was Nodal, a protein in the TGF-β superfamily (Liu *et al.*, 2015). When pregnant mice were given the HDAC inhibitor Trichostatin A (TSA) their offspring were found to have reduced levels of neurogenesis and increased progenitor proliferation in the striatum (Shakèd *et al.*, 2008). The mechanism of action proposed was through the inhibition of BMP2/4 signalling. HDAC1 and HDAC2 can compensate for each other (Montgomery *et al.*, 2009) indicating they can perform the same functions, however, they do display some differences. Deletion of only HDAC2 using GLAST:CreERT2 found it had a specific role in adult neurogenesis. HDAC2 was required in the progenitor for full differentiation and survival of neurons made at this time (Jawerka *et al.*, 2010).

Other studies have found in contradiction to these results that HDAC has a negative role in neurogenesis. TSA treatment during pregnancy increases neurogenesis in the cortex of the subsequent offspring and promotes neurogenesis of cortical progenitors *in vitro* (Shakèd *et al.*, 2008). Treatment with the HDAC inhibitor valproic acid (VPA) decreased granule cell proliferation in the dentate gyrus of adult rats and increased the numbers of new born neurons ($BrdU^+/Tuji1^+$) (Hsieh *et al.*, 2004). HDAC1 knockout in mESCs increased gene expression of mesodermal and ectodermal markers including neural (nestin and βIII-tubulin) (Dovey, Foster and Cowley, 2010). Numerous studies using progenitor cells from rodents found that treatment with HDAC inhibitors promoted neurogenesis while decreasing gliogenesis through upregulation of neurogenic genes such as NeuroD (Hsieh *et al.*, 2004; Balasubramaniyan *et al.*, 2006; Siebzehnrubl *et al.*, 2007; Yu *et al.*, 2009). HDAC inhibitor treatment *in vivo* or *in vitro* was found to inhibit the ability of cells from the SVZ to form neurospheres (Zhou *et al.*, 2011; Foti *et al.*, 2013). In the chick embryo the expression of different chromatin remodelling enzymes is downregulated as neural differentiation occurred including HDAC1. HDAC1 expression in this context was regulated by FGF signalling (Olivera-Martinez *et al.*, 2014). Neural differentiation of human mesenchymal stem cells is enhanced by treatment with VPA (Ehashi *et al.*, 2014; Talwadekar *et al.*, 2017).

There are several possible reasons for the contradiction of these results. Firstly, differences may be due to the difference in pharmacological and genetic approaches. Some authors suggest that pharmacological inhibition is more transient and incomplete leading to the survival of some neuronal precursors whereas a genetic

approach is more robust and complete (Montgomery *et al.*, 2009). Pharmacological inhibition will also target multiple HDACs which may have different roles in neurogenesis. HDAC1 depletion in mESCs inhibited neural differentiation whereas another Class I gene, HDAC3 depletion had the opposite effect (Liu *et al.*, 2015). The compounds also will target every cell in the lineage. This is an important consideration as HDAC1 functions in the ERGs may be different to function in the neurons. Secondly, the location of the cells used in the experiments may lead to different outcomes as other cues or epigenetic factors that the cells have been exposed to could alter their behaviour. This is particularly evident in the study where TSA treatment, in the same animals, led to a decreased neurogenesis in the striatum but an increase in the cortex (Shakèd *et al.*, 2008). Therefore, studies involving cell type specific manipulations of HDAC1 are necessary to resolve the issues that have been raised by previous research to date.

1.4.5 HDAC1 in the development of the nervous system in zebrafish

Studies that have investigated role of Hdac1 in neurogenesis in fish have often used mutants of Hdac1 (Cunliffe, 2004; Stadler *et al.*, 2005; Yamaguchi *et al.*, 2005; Harrison *et al.*, 2011) which have global effects. These mutants are embryonic lethal and fish do not develop past early stages. These fish have deficits in many organs. In the nervous system there is reduced expression of important proneural transcription factors e.g. *neurod, ascl1b, lhx9, dlb* (Harrison *et al.*, 2011). Using chromatin immunoprecipitation Hdac1 was found to bind to the promoter of *ascl1b* showing direct evidence of its role in modulating expression of this transcription factor. Expression of important transcription factors involved in development of the nervous system are altered in the *hdac1* mutants; *olig2* is abolished while *nkx2.2* and *pax6* are increased. The expression of *sox2* is expanded suggesting that the progenitors are failing to differentiate correctly without Hdac1 (Cunliffe and Casaccia-Bonnefil, 2006). The *hdac1* mutants have reduced neuron numbers and abnormally arranged glial cells, as determined by Hu and GFAP immunohistochemistry. In the spinal cord and hindbrain, the mutants have fewer isl1-positive motor neurons. These animals also have a reduced capacity to respond to hedgehog signalling and do not make more motor neurons when treated with hedgehog as expected (Cunliffe, 2004). The numbers of mature oligodendrocytes and oligodendrocyte precursor cells are also reduced (Cunliffe and Casaccia-Bonnefil, 2006). The mutants have reduced proliferation levels in the hindbrain at earlier stages of development which then return to wildtype levels (Cunliffe, 2004). In the retina of *hdac1* mutants, however, the

progenitors proliferate more and fail to differentiate into neurons (Stadler *et al.*, 2005; Yamaguchi *et al.*, 2005). Cyclin genes (cyclin D and E) expression was increased in the mutant retina showing that Hdac1 normally represses these genes to allow the progenitors to exit the cell cycle (Stadler *et al.*, 2005). Signalling pathways are also impacted in the *hdac1* mutants. There is increased expression of notch target genes e.g. *her4, her6* (Cunliffe, 2004; Harrison *et al.*, 2011) and the canonical Wnt signalling (Yamaguchi *et al.*, 2005) is enhanced, indicating that Hdac1 inhibits these pathways. In the hindbrain rhomoberes the expression of *fgf20a* and the FGF-regulated gene *erm* are dependent on Hdac1 function (Lightman, Harrison and Cunliffe, 2011). CRISPR targeting of Hdac1 in zebrafish found the same neurogenic phenotypes, with mutants having reduced numbers of neurons (HuC^{+}) (Schultz *et al.*, 2018). They also found that larvae with somatic CRISPR targeting of Hdac1, showed the appearance of condensed chromatin in the neural stem cells in the telencephalon suggesting the cells were quiescent. Treatment of zebrafish with HDAC inhibitors such as TSA and VPA, during embryogenesis show the same phenotypes as the mutants (Yamaguchi *et al.*, 2005; Harrison *et al.*, 2011). HDAC inhibitors reduced the constitutive neurogenesis that occurs in the adult zebrafish optic tectum (Dozawa *et al.*, 2014).

All together the studies in zebrafish point to Hdac1 having a positive role in neurogenesis. The differences in the amount of proliferation between the retina and other CNS regions suggest that Hdac1 may exert different roles depending on the location of the cells. In summary, Hdac1 supports the formation and subsequent differentiation of neural precursors in zebrafish development.

1.5 Summary

Previous research in the group has found that neurogenic developmental signalling pathways are often redeployed in regeneration. Successful regeneration requires the complex interplay of signalling pathways and transcriptional control mechanisms. Due to the positive role of Hdac1 in neurogenesis during development, we predict that Hdac1 would have a positive role in neuroregeneration in the zebrafish spinal cord and that the inhibition of Hdac1 would conversely inhibit this regeneration. I hypothesise that Hdac1 upregulation is a mechanism that the zebrafish spinal cord ERG uses to enable the integration of numerous extrinsic signals which leads to the expression of a programme of genes involved in successful neuroregeneration. To address this, I had two aims of my thesis.

Firstly, in chapter 3 I generated a system to manipulate Hdac1 levels in a cell specific manner. The contradictory mammalian results investigating HDAC1 in neurogenesis highlights the necessity for such an approach. There are numerous methods that can be used to achieve this; Cre-Lox, Heatshock, Gal4-UAS, CRISPR and the Tet-On system. After considerations the Tet-On system was chosen due to its ability to provide spatial and temporal control of gene expression. I used the Tet-On system to express either a dominant negative form of Hdac1 (dnHdac1) or wildtype Hdac1 in zebrafish spinal cord ERGs. I characterise the expression pattern of these lines, the dynamics of the expression and their functionality.

Secondly, in chapter 4 and 5 I used the newly generated genetic tools to investigate the role of Hdac1 in neuroregeneration in the zebrafish spinal cord. I found that the expression of dnHdac1 in ERGs inhibited neuroregeneration. The expression of dnHdac1 in the ERGs of the pMN domain reduced their injury induced proliferation. I found that augmenting Hdac1 activity after a lesion did not lead to a further increase in neuroregeneration but that in the unlesioned spinal cord led to increased proliferation of spinal cord ERGs. Inhibition of acetylation was found to be sufficient to promote neurogenesis in the unlesioned larval spinal cord.

Thus, Hdac1 acts as a positive regulator of neuroregeneration in the zebrafish spinal cord and may be an initial signal that moves the ERGs from relative quiescence into an active state.

Chapter 2 Materials and Methods

2.1 Zebrafish Techniques

2.1.1 Zebrafish husbandry

All fish were raised and housed according to standard conditions (Westerfield, 2007), at temperature of 26.5℃ in 14/10 hour light dark cycles. Embryos were kept at 28.5℃ in conditioned aquarium water with or without 0.0001% methylene blue. For the purpose of this study I used the following lines described in the Table 1 below. Male and female animals were used.

Table 2-1: Fish lines used in the study with abbreviations and references

Line name	**Abbreviated as**	**Reference**
Wildtype	WIK	
Mnx1:GFP	*Hb9*:GFP	(Flanagan-Steet *et al.*, 2005)
Mnx1:TagRFP-T	*Mnx1*:RFP	(Jao, Appel and Wente, 2012) obtained from Professor Aristides Arrenberg
Olig2:DsRed2	*Olig2*:DsRed	(Kucenas *et al.*, 2008)
Her4.3:irtTAM2(3F)-p2a-AmCyan	*Her4.1*:TetA	(Wehner *et al.*, 2014)
Ubiquitin:irtTAM2(3F)-p2a-AmCyan	*Ubiquitin*:TetA	(Wehner *et al.*, 2014)
Mpeg1:GFP	*Mpeg1:GFP*	(Ellett *et al.*, 2011)

As part of my PhD study I also generated two new transgenic lines for Tet-On system manipulations. I generated Tet responder lines that expressed wildtype Hdac1 or dominant negative Hdac1 under the control of the Tet Responder element. Single transgenic animals were generated and then subsequently breed with driver lines. Double transgenic embryos were raised to perform crosses with additional lines and adult lesions.

2.1.2 Generation of zebrafish transgenic lines

Plasmid DNA (50ng/µl) along with Tol2 transposase mRNA (25ng/µl) was injected into single cell stage of WIK embryos with an air pressure driven microinjector. Once adult, injected fish were screened for founders by breeding with Tet Activator lines. Founders were selected for amount and selectivity of expression. Once a line was

generated both single and double transgenic sublines were kept. Double transgenics were screened by doxycycline treatment after 3 days post fertilisation (dpf) to avoid any developmental effects of transgene induction.

2.1.3 Spinal cord lesions

Larval: Animals at 3 dpf were anaesthetised with MS-222. They were placed on agar plate and placed in a lateral position. Lesions were performed using a sharp 30 gauge injection needle. Care was taken to not hit the notochord. Larval were placed back into conditioned water to recover before addition of drug treatments.

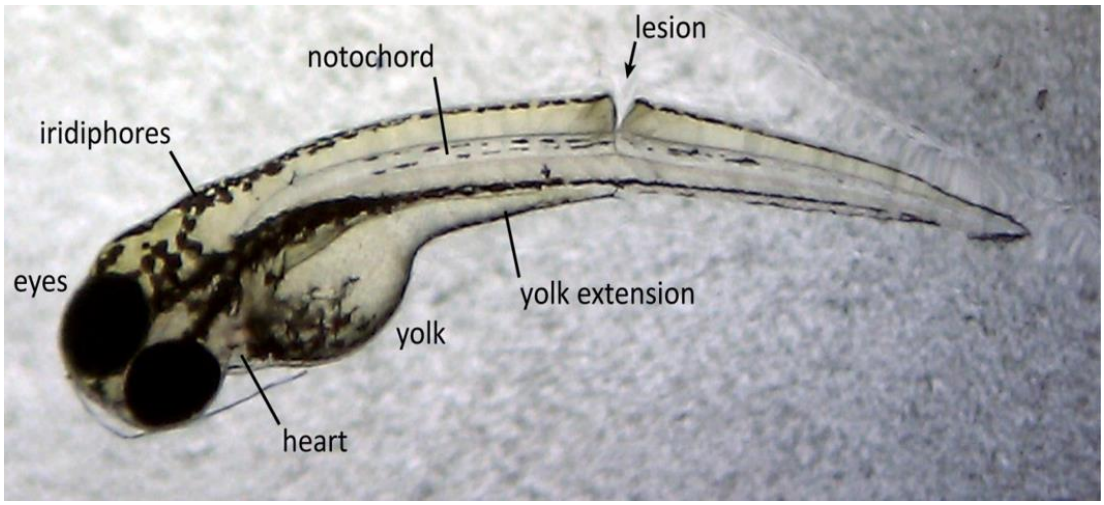


Figure 2-1: Larval lesion. A zebrafish larvae at 3dpf with lesion in dorsal trunk area, at the level of the end of the yolk extension. Picture taken from (Ohnmacht *et al.*, 2016) .

Adult: Animals between 4 and 9 months of age were used for spinal cord injuries. A week prior to surgery animals were placed in single housing to ensure optimal health and placed at high salinity 1200µS to reduce of bacterial infection. The procedure for the surgery is published previously (Becker *et al.*, 1997). Briefly fish were anaesthetised in 0.02% MS-222 in PBS and placed on ice. A longitudinal incision was performed on side of fish to expose the vertebral column. The spinal cord was completely transected under visual control 3.5 mm caudal of the brainstem. The surgeries were performed by Dr. Thomas Becker. After surgery they were placed into water containing antibiotic/antifungal agent eSHa 2000 (125µl into 10L cH_2O) to prevent infection and reduce risk of accidental death. They are also kept in dark for first 3 days post lesion to prevent excessive movement.

2.1.4 Larval drug treatments

All drug applications were done after injury (3dpf). After the lesion larval were placed into 6 well plate of 15 fish/well in a total solution volume of 5ml. Final concentrations of drug were as follows; Mocetinostat at 1µM, Trichostatin A at 200nM, EML425 at 3µM, Lipopolysaccharide at 50µg/ml, Pomalidomide at 170µM, Doxycycline at 485nM. All were dissolved in DMSO apart from Doxycycline and Lipopolysaccharide which are in conditioned water. Larvae were left for 2 days to regenerate. EdU (5-ethynyl-2-deoxyuridine) at final concentration of 50µM was added to the 5ml at timepoints required depending on the experiment. The same procedure and timeline was used for the unlesion experimental groups. At 5dpf animals were fixed in 4% PFA for 3 hour at room temperature (RT) or 4℃ overnight and processed for EdU detection and immunohistochemistry.

2.1.5 Adult drug treatments

Subjects were randomly assigned to treatment groups using Graphpad randomise function (https://www.graphpad.com/quickcalcs/randomize1/). Doxycycline was added at 25µg/ml in 1L to fish water.

2.1.6 Fin Clipping for genotyping of adult transgenic fish

Fish were briefly anaesthetised in 0.02% MS-222 solution. Approximately 2mm of the caudal fin was cut off with a scalpel and put into tubes containing lysis buffer (2.5µl of Proteinase K at 20mg/ml, 10µl Taq Buffer, 90µl dH_2O). Tubes were placed into a thermocycler for 20minutes at 65℃. After vortexing, the tubes were placed for an additional 20minutes at 95℃. After 2 minutes centrifugation, 40µl of the supernatant was removed. This was used for subsequent PCR and stored at -20℃.

2.2 Molecular techniques

2.2.1 Cloning strategy for zebrafish Hdac1

Wildtype Hdac1 primers (shown in Table 2) used to clone Hdac1 from zebrafish cDNA. Underlined sequences correspond to the end and the beginning of Hdac1. Sequences for restriction sites added for SmaI and XhoI restriction sites were included (marked in red).

Table 2-2: Hdac1 primers

Forward (5'-3')	CCC GGA ATG GCG CTG AGT TCT CAA GG
Reverse (5'-3')	AAT AAC TCG AGT CAC ACT GTT TTT AAT TCT TCT TTT GG

The resulting 1.5kB fragment was digested using SmaI and XhoI restriction enzymes and ligated into the similarly digested YFP containing vector to form a N- terminus tagged YFP-Hdac1. Touch up PCR was then used to introduce MluI and BglII restriction enzyme sites to enable digestion and ligation into the TetRE vector. Primers used are shown in Table3, underlined corresponds to beginning and end of YFP-Hdac1 sequence and restriction enzymes are shown in red.

Table 2-3: Primers to add on MluI and BglII restriction sites

Forward (5'-3')	TTATTTACGCGTCCACCATGGTGAGCAAGGGCGAGGA
Reverse (5'-3')	TTTATTAGATCTAAAAAACCTCCCACACCTCCCCCTGAACCTC

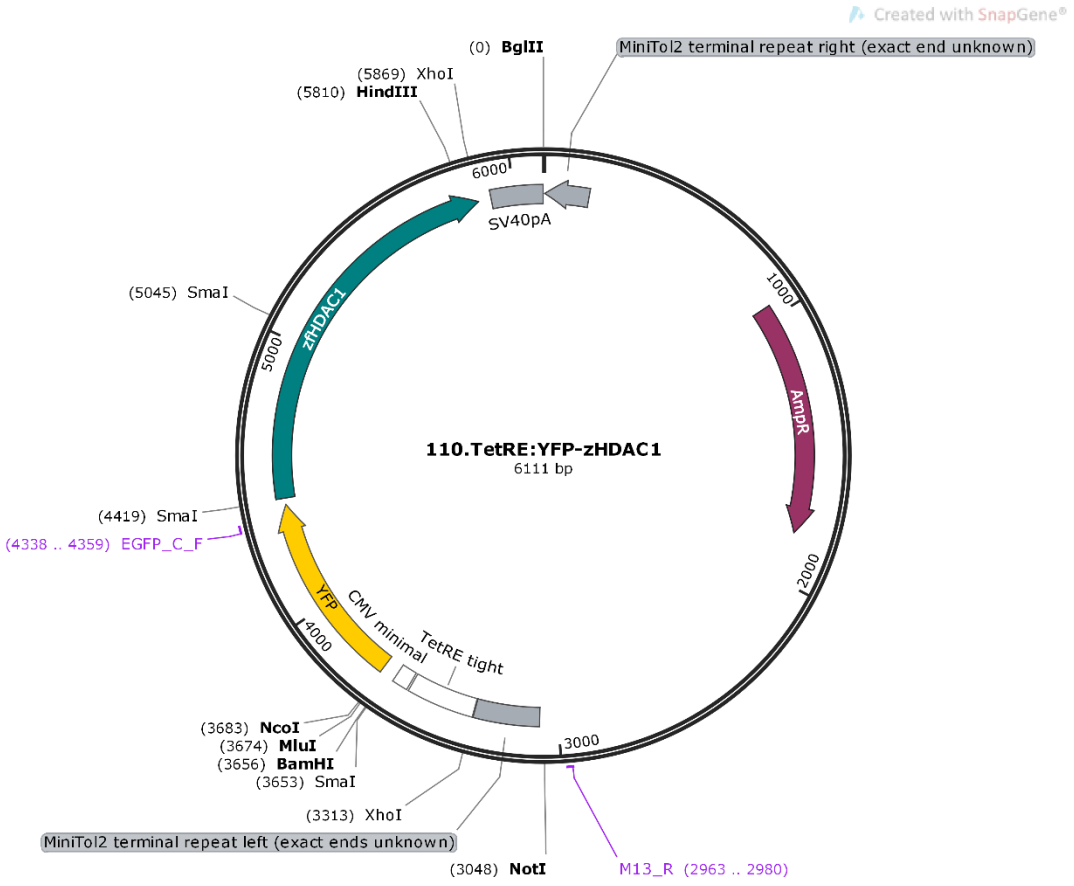


Figure 2-2: Plasmid map of final TetRE: YFP-Hdac1 plasmid. Main features of the plasmid are displayed. The restriction enzymes and sequencing primers used in the study are shown. Generated with SnapGene.

2.2.2 Site directed mutagenesis

Dominant negative Hdac1 primers were designed on Primer X (http://www.bioinformatics.org/primerx/) to induce the CA to GC change at position 141 in the zebrafish Hdac1 sequence (Table 4). The two base pair change is marked in red, flanked by 17/18 base pairs.

Table 2-4: DnHdac1 primers with GC change at position 141

Forward (5'-3')	GGGCAGGAGGTCTACATGCTGCTAAGAAATCAGAGGC
Reverse (5'-3')	GCCTCTGATTTCTTAGCAGCATGTAGACCTCCTGCCC

1. Two Q5 polymerase PCR using TetRE:YFP-Hdac1 as a template to generate two fragments that will overlap and contain the mutation site.
   Forward Primer (Table 3) + Reverse DnHdac1 (Table 4)
   Reverse Primer (Table 3) + Forward DnHdac1 (Table 4)
2. Two fragments (1µl each) from step 1 annealed to generate new template

Table 2-5:Annealing protocol

Temperature (℃)	**Time**	**Cycles**
98	30s	1
98	10s	15
58	20s	15
72	30s	15
72	1minute	1

3. Fusion PCR with original primers (Table 3) with new template from step 2 to generate the full YFP-dnHdac1 fragment with the two base pair change.
4. MluI and BglII digestion and ligation into the TetRE vector.

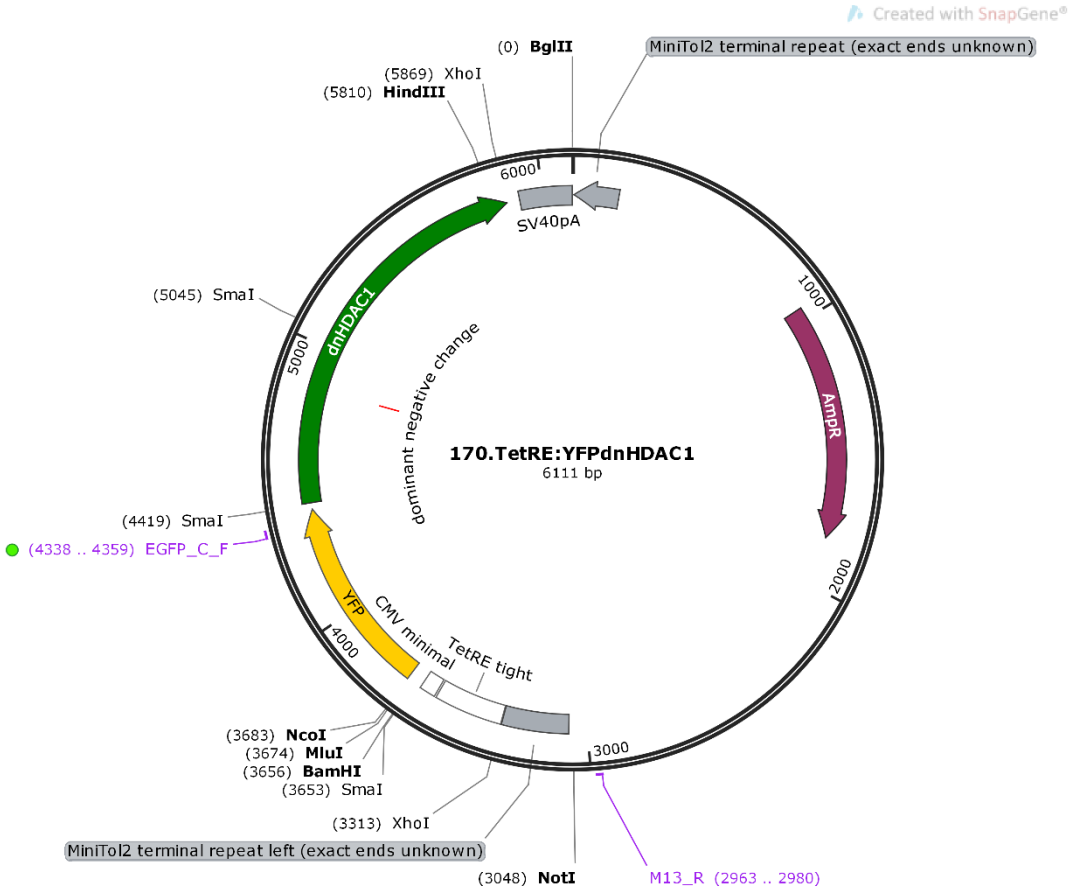


Figure 2-3: Plasmid Map of TetRE:YFP-dnHdac1. Main features of the plasmid are displayed. The restriction enzymes and sequencing primers used in the study are shown. Generated with SnapGene.

2.2.3 RNA extraction

Dechorionated embryos at the required age were collected and extracted according to the manufacturer's instructions following 'protocol for extraction of total RNA from animal tissues' from RNeasy Mini Kit (Qiagen: 74106). Samples were suspended in 1% β-mercaptoethanol solution and vortexed for 5 minutes. Once homogenised an equal volume of 70% Ethanol was added. At the end of the protocol RNA was eluted in 30µl of RNAase free water. Concentrations and purity was measured on a nanodrop. RNA was then stored at -80℃.

2.2.4 cDNA extraction

RNA from the extraction was used to generate relevant cDNA according to the manufacturer's instructions from iScript cDNA synthesis kit (Biorad:1708890). RNA was diluted to final amount of 1µg if necessary. Reaction protocol was as follows: Priming 5min at 25℃, Reverse transcription 20min at 46℃, Inactivation 1 min at 95℃.

2.2.5 Standard Polymerase Chain Reaction (PCR)

Reactions were made up using either NEB Taq DNA polymerase (M0273) for standard PCR or Q5 High-fidelity DNA polymerase (M0491) used for sensitive PCR. Master mix was made up according to the manufacturer's guidelines. The annealing temperatures were calculated on NEB Tm Calculator (https://tmcalculator.neb.com/#!/main). Gradients of annealing temperatures were used if needed. The thermocycler was set up to the following conditions depending on the length of product and the Tm of the primers.

Table 2-6: Standard Taq Polymerase protocol

Steps	**Temperature (℃)**	**Time**	**Cycles**
Initial denaturation	95	30s	1
Denaturation	95	15-30s	30
Annealing	45-68	15-60s	30
Extension	68	1min/kb	30
Final extension	68	5minutes	1
Hold	4	∞	1

Table 2-7: Q5 Polymerase protocol

Steps	**Temperature (℃)**	**Time**	**Cycles**
Initial denaturation	98	30s	1
Denaturation	98	5-10s	30
Annealing	50-72	10-30s	30
Extension	72	20-30s/kb	30
Final extension	72	2minutes	1
Hold	4	∞	1

Touch up PCR was used in the generation of some fragments which required a step wise increase in annealing temperatures; 7 cycles with 68℃ annealing temperature and then 28 cycles increased up to 72℃.

Colony PCR was used to screen through possible positive colonies containing the dnHdac1 fragment. Colonies were picked using a pipette tip and placed into PCR tube containing 10µl of dH_2O and then processed with standard PCR protocol.

2.2.6 Quantitative PCR (qPCR)

QPCR reactions were set up in a 96-well plate. The reaction solution including cDNA sample was prepared according to manufacturer's protocol from SSoAdvanced Universal SYBR Green Supermix (BioRad:1725270) and distributed into the wells. Each sample was done in triplicate. The well plates are then sealed with clear seal using a roller to ensure proper attaching. The whole plate was spun down before putting it into the reaction block of the Roche LightCycler 96 and running the qPCR program (Table 8).

Table 2-8: qPCR protocol

Steps	**Temperature (℃)**	**Time**	**Cycles**
Preincubation	95	10mintes	1
Denaturation	95	10s	42
Annealing	60	20s	42
Extension	72	20s	42
Melting	60-95	10-60s	1

Following primers were used in qPCR reaction.

Primer	**Forward (5'-3')**	**Reverse (5'-3')**
β-actin	CACGAGAGATCTTCACTCCCC	TCCCATGCCAACCATCACTC
Hdac1	TGTCCGAGTACAGCAAGCAG	TGATGTAGACCTCCTGCCCA

2.2.7 Gel electrophoresis and purification

Horizontal agarose gel electrophoresis was performed to analyse the outcome of restriction digests and PCR reactions. The fragments were run out in 1% agarose gels with 0.0005% Gel Red (Biotium:41003) in 1xTAE buffer. To load the samples, 6X DNA loading buffer (B7025S) was added to DNA samples to a 1X fold final concentration. The products were separated in a Bio-Rad electrophoresis chamber filled with 1xTAE running a current at approximately 100V. Samples were run with DNA ladders (N0468S; N0551S) depending on predicted size of fragments. The time of separation was dependent on fragment size. When necessary bands of appropriate size were cut out using UV lamp and then purified with QIAquick Gel Extraction kit (Qiagen:28706) according to manufacturer's instructions.

2.2.8 Ligation and bacterial transformation

Ligation was performed using T4 DNA ligase (M0202). Reaction mix was made up according to manufacturer's instructions. For optimal ligation efficiency, inserts and vector DNA were combined at a molar ratio of 3:1. Reaction was left at 16℃ overnight. It was heat inactivated for 65℃ for 10 minutes. The product was then used for transformation.

Vectors were transformed into NEB 5-*alpha* Competent *E.coli* (C2987H) according to the high efficiency transformation protocol. Cells were thawed on ice and 1-5µl of vector was added. Mixture was left on ice for 20 minutes then following by 40 second heat shock at 42℃. Bacteria left to grow in LB agar at 37℃ for at least 1 hour for ampicillin and for 1hour and half for kanamycin before plating on the appropriate antibiotic plate. Plates were left at 37℃ overnight.

2.2.9 Bacterial Cultures

Colonies from plates were picked using pipette tip. It was added to 3ml in 15ml blue cap tube (mini prep) or 50ml in conical flask (midi prep) of LB agar containing 100µg/ml ampicillin or 50µg/ml kanamycin. Cultures were left overnight shaking on an orbital shaker at 37℃.

2.2.10 Plasmid Isolation

Plasmids were purified from 3ml and 50ml cultures using QIAprep Spin Miniprep Kit (Qiagen:27106) and QIAGEN Plasmid PlusMidi Kit (Qiagen:12943) preparation kits respectively. Samples were analysed on a nanodrop for DNA concentration and purity.

2.2.11 Sequencing

5ul of samples at 100ng/µl were sent to Source Bioscience for sanger sequencing (https://www.sourcebioscience.com/services/genomics/sanger-sequencing-services/) . Primers were selected according to the sequence of the vectors; EGFP_C_F selected for dnHdac1 mutation site and M13_R for TetRE site (shown on plasmid maps Figure 1.1 and 1.2).

2.2.12 Restriction Digest

The appropriate NEB enzymes were used according to manufacturer's instructions.

2.2.13 Protein Extraction

Larvae (60 embryos per 200µl) were put into lysis buffer (1% Triton-X,1xPBS, Protease Inhibitor Cocktail Tablet (Roche:04693159001). Samples were homogenised with a pestle on ice and put on rotator at 4℃ for 1 hour. After a

centrifugation at 12000rpm for 20 minutes at 4℃ the supernatant was removed. This stored at -20℃ until use.

2.2.14 BCA assay

Quantification of total protein levels after extraction was performed by using Pierce BCA Protein Assay Kit (Thermo scientific:23227) following the manufacturer's protocol for the microplate procedure. Diluted albumin (BSA) standards was prepared which ranged from 2000µg/ml to 25µg/ml. Standards and samples were arrayed into 96 well plate and incubated with BCA working reagent. Plate was left at 37℃ for 30minutes. Plate was cooled to RT for at least 15minutes. The absorbance at 562nm was measured on a plate reader. The measurements from the BSA standards were used to generate a standard curve from which the protein concentration of the samples could be determined.

2.2.15 Western blotting

Using the protein concentration from the BCA assay equal amounts of protein for each sample was combined in a 3:1 ratio with 4X Laemmli Sample buffer (BioRad:1610747) mixed with β-mercaptoethanol. Samples were boiled for 5minutes at 95℃. Samples were loaded alongside a PageRuler Plus Prestained Protein Ladder (26619) in 12% SDS-PAGE gel.

Table 2-9: Recipe for SDS-PAGE gel

12% Separating Gel	**4% Stacking Gel**
6.9ml ddH_2O	6.3ml ddH_2O
4.8ml 40%Acrylamide	1ml 40%Acrylamide
4ml 1.5M Tris pH8.8	2.5ml 0.5M Tris pH6.8
160µl 10% SDS	100µl 10% SDS
160µl 10%APS	100µl 10%APS
16µl TEMED	10µl TEMED

Samples were run at 80V in 1X TGS buffer (BioRad:1610732) until the dye front reached the bottom of the gel. The gel was removed and sandwiched with nitrocellulose membrane 0.45 µm (BioRad:1610115) between sponges and filter paper (BioRad:1703932) in the below configuration.

Figure 2-4: Configuration of Transfer sandwich. From https://www.abcam.com/protocols/general-western-blot-protocol.

The sandwich was submerged in transfer buffer (1X TGS buffer with 20% methanol) in the transfer cassette and ran for 60minutes at 100V. A cooling unit and buffer was constantly stirred to maintain buffer temperature and ion concentration during transfer.

After transfer the membrane was stained with Ponceau S solution for 5minutes on shaker. This was washed off with water and protein bands were observed to ensure proper protein transfer. The membrane was blocked in 5% milk (Santa Cruz biotechnology:sc-2324) in 0.1%PBS-Tween20 (PBSTw) for 1 hour at RT. Primary antibodies were diluted in 5% milk and incubated with membrane at 4℃ overnight. After washes with 0.1% PBSTw, the membrane was incubated with fluorescent secondary antibodies diluted in 5% milk for 45 minutes at RT. The membrane was washed with 0.1% PBSTw and then imaged using LiCor Odyssey machine. The signal intensity of Ac-H4 between samples was normalised to signal intensity of the standard (alpha-tubulin).

2.3 Histology

2.3.1 EdU detection

Following procedure was adapted from (Kimmel and Meyer, 2010) using the Click-iT EdU Imaging Kit (Invitrogen: C10340). After PFA fixation larvae were transferred to methanol and placed at -20℃ for at least 2 hours. After rehydration and several washes in 0.1% PBS-Triton X (PBSTx) larvae were incubated in Proteinase K (Invitrogen:25530049) at 10µg/ml for 45 minutes. After 15 minutes refixation in 4% PFA larvae were washed in 0.5%PBSTx/1% DMSO/PBS for 20 minutes. After wash in 0.1%PBSTx the larvae were put in freshly prepared Click-iT reaction cocktail

overnight at 4℃ or 3 hours at RT. The cocktail was removed and after several PBSTx washes the larvae were processed for immunohistochemistry.

Table 2-10: Click-iT reaction cocktail

Component	**Amount(µl)**
10x Click-iT Reaction Buffer	44
CuSO2	10
Fluorescent dye azide	2.5
10x Reaction Buffer additive	5
ddH2O	439

2.3.2 IHC on wholemount larvae and larval sections

Wholemount larvae or sections were incubated in Donkey Blocking buffer (1% DMSO, 1%BSA, 1% normal donkey serum, 0.7% Triton-X) for at least 1 hour. Primary antibodies were diluted in blocking buffer and left for 2 nights at 4℃. After washes in 0.1% PBSTx, the appropriate secondary antibodies were diluted in blocking buffer and left for 1 night at 4℃. Following washes in PBStx and PBS larvae were cleared in 70% glycerol and mounted for imaging.

2.3.3 Harvest of adult tissue

Adult fish were terminally anaesthetised in 0.1% MS-222. The heart was exposed and a needle attached to a syringe was inserted into the bulbus arteriosus. Blood was flushed out with PBS followed by 4% PFA to fix the tissue. The fish were left in 4%PFA at 4℃ overnight. The spinal cord was dissected and washed in PBS.

2.3.4 Vibratome sectioning

The adult spinal cord or whole zebrafish larvae were embedded in 4% Agar in PBS. Pyramidal blocks were prepared and coronal sections were cut on a vibratome HM-650V (Microme-Germany). Sections were between 50-100 µm thick. The sections were collected into 24-well plate containing PBS.

2.3.5 Floating IHC for adult spinal cord sections

Vibratome sections were places in 24 well plate in PBS. Antigen retrieval was performed with citric acid (10mM, pH6) at 80℃ for 30 minutes. The sections were washed in 50mM glycine in 0.1%PBSTx for 10 minutes to remove free aldehydes that could introduce background noise. After 0.1%PBSTx washes sections were blocked in 2% normal donkey serum (NDS) for at least 1 hour. Primary antibodies were diluted

in 2% NDS and incubated with sections at 4℃ overnight on shaker. After several washes in 0.1%PBSTx the sections were incubated in the appropriate secondary antibodies were diluted in 2% NDS for at least 45 minutes on RT shaker. This was washed off with 0.1%PBSTx and PBS and sections cleared with 70% glycerol.

2.3.6 Stereological counting

In wholemount larvae measurements were taken from confocal image stacks through the complete thickness of the spinal cord of 3 segments (approx. 250µm in length) in the midthroaic region (above the end of the yolk sack).

In lesioned animals, confocal image stacks through the complete thickness of the spinal cord were taken that covered the entire lesion site (black box in Figure2.5A). The interval on the z-stack was set at 1µm thick, each fish was typically ~30 sections. This image was then cropped in Image J to a rectangle (red box in Figure2.5B) that was centred on the lesion site (white dashed lines in Figure2.5B) and was 250µm in length and the height of the entire trunk. The cropped region was analysed through the entire z-stack looking at each individual slice to quantify the number of double positive cells. Image J was used to view the channels both in combination and separately to ensure accurate counting. For all experiments the observer was blinded to experimental conditions.

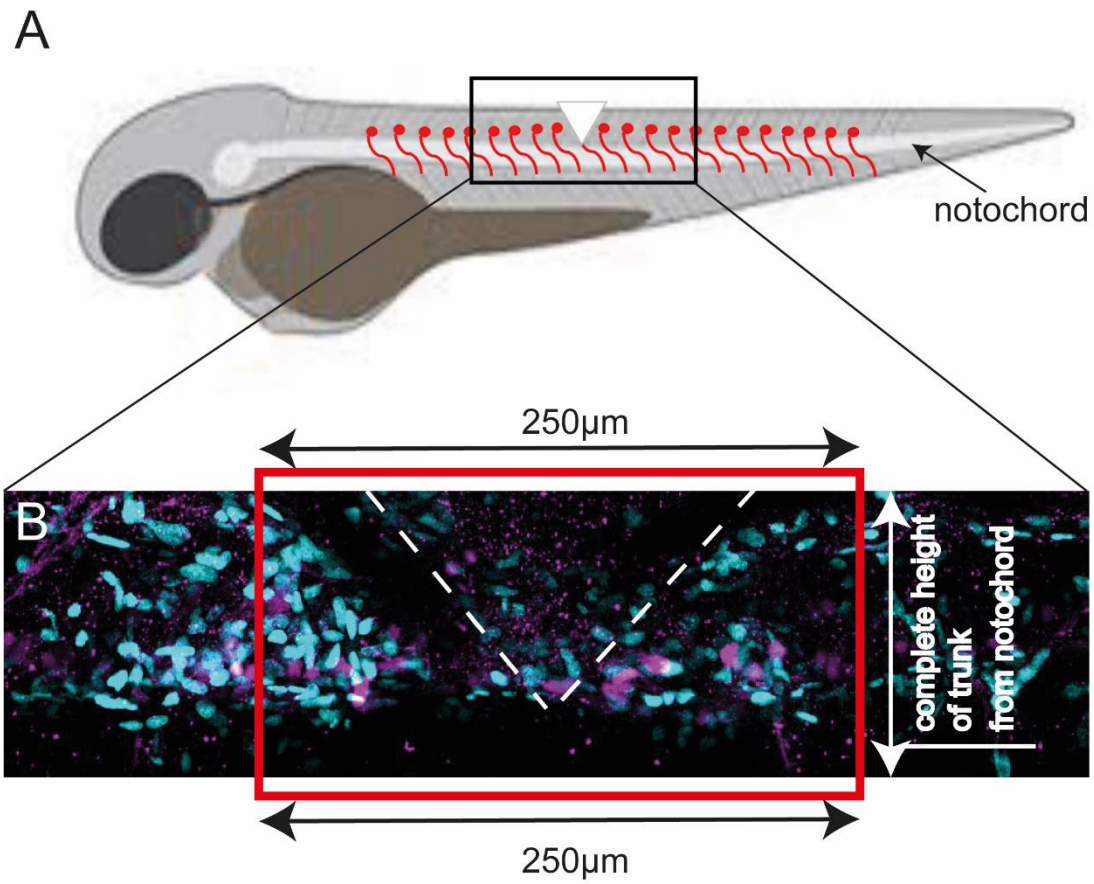


Figure 2-5:Region in wholemount larvae used for cell quantifications after injury. **A**; Lateral view of a schematic of whole zebrafish larvae showing the region that was imaged on the confocal (black box). **B**; Maximal intensity projection of confocal image of wholemount larvae taken within the region showed in black box in A. Red box shows the cropped region that was used for quantification. Cropped region was rectangle of 250µm width and complete height of trunk. White dashed line marks boundaries of the lesion site.

In adult spinal cord vibratome sections, 3 randomly selected sections from 750µm either side of the injury were counted, normalised to number of sections and averaged. Cell numbers were calculated from this for the entire 1.5mm surrounding a lesion site. All Hb9 positive cells across the entire dorso-ventral and medial-lateral planes were counted (example of staining shown in Figure 2.6E). For PCNA counts, all ventricular PCNA positive cells were counted (example of staining shown in Figure 2.6F). The cells that were visible in the last optical section were excluded from final count. For all experiments the observer was blinded to experimental conditions.

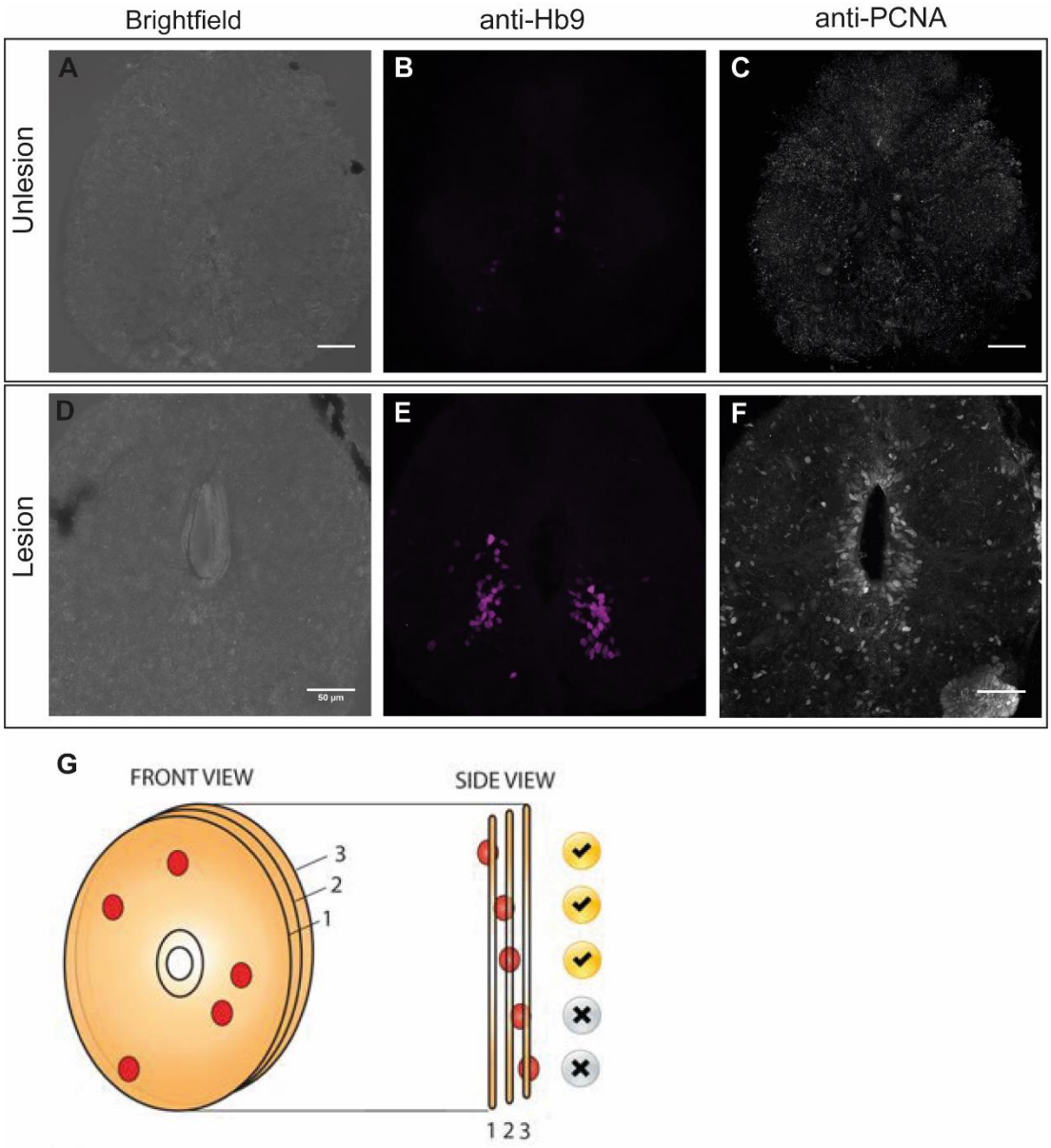


Figure 2-6: Quantification of adult regeneration was performed with Hb9 and PCNA immunohistochemistry on vibratome sections. **A-C**; Maximal intensity projections of vibratome section from unlesioned spinal cord showing brightfield (A), anti-Hb9 (B) and anti-PCNA (C). **D-F**; Maximal intensity projections of vibratome section from lesioned spinal cord showing brightfield (D), anti-Hb9 (E) and anti-PCNA (F). **G**; Schematic representation of cell counts in spinal cord sections. All cells included in counts, except those located in the final optical section. From (Barreiro-Iglesias *et al.*, 2015).

2.4 Image acquisition and data analysis

For acquisition of fluorescent images a LSM 710 and 880 confocal microscope operating Zeiss Zen software was used. Image analysis was performed using Image J. Preparation of figures was done on Adobe Illustrator CC 2018.

Statistical analysis was performed with Graphpad Prism v7. Data was tested for gaussian distribution using Shapiro-Wilk normality test. The result of this determined whether parametric (Unpaired T-test, ANOVA) or nonparametric (Mann-Whitney, Krustal-Wallis) tests were used. Power analysis was performed by G*Power 3.1.9.2; Post hoc analysis performed on statistically significant data to compute the achieved power and A priori analysis to determine the required sample size of non significant data to reach alpha of 0.05 and power 0.08.

QPCR data was analysed with Roche LightCycler 96 SW1.1 software. Western blots were analysed using ImageStudio Lite Version5.2. Sequence analysis was performed with Vector NTI and SnapGene. Resources for sequence data was NCBI database (https://www.ncbi.nlm.nih.gov). Zebrafish specific information was taken from The Zebrafish Information Network (http://zfin.org/).

This thesis was compiled on Microsoft Word 2016, all references were managed and imported using the free citation software Mendeley Desktop and the provided Word plug-in.

2.5 Materials

2.5.1 Primary Antibodies

Antigen	**Species**	**Source**	**Catalogue number**	**Dilution**
α-Acetyl-histone H4	rabbit	Abcam	ab177790	1:1000
α-Alpha-tubulin	mouse	DSHB	12G10	1:2000
α-GFP	chicken	Abcam	ab13970	1:200
α-Hb9 (MNR2)	mouse	Developmental Studies Hybridoma Bank (DSHB)	81.5C10	1:400
α-HuC/HuD	mouse	ThermoScientific	16A11	1:100
α-Proliferating Cell Nuclear Antigen (PCNA)	rabbit	Abcam	ab18197	1:1000
α-phospho-Histone H3 (pH3)	rabbit	Millipore	06-570	1:1000

2.5.2 Secondary Antibodies

Name	**Catalogue number**	**Source**
Alexa 488 conjugated donkey anti-chicken IgG(H+L)	703-545-155	Jackson
Cy3 conjugated donkey anti-mouse IgG (H+L)	715-165-150	Jackson
Cy5 conjugated Donkey anti-rabbit IgG (H+L)	711-175-152	Jackson
IRDye 800CW Goat anti- mouse	925-32210	LiCor
IRDye 680LT Goat anti- rabbit	925-68021	LiCor

2.5.3 Chemicals reagents and products

Name	**Catalogue number**	**Source**
Acrylamide:Bis-Acrylamide	BP1406	Fisher BioReagents
Agarose	BPE1356	Sigma-Aldrich
Aminobenzoic acid ethylmethylester (MS-222)	A5040	Sigma-Aldrich
Ampicillin	A9518	Sigma-Aldrich
Ammonium Persulfate (APS)	201531000	Acros Organics
β-mercaptoethanol	63700	Fluka
Bovine serum albumin (BSA)	A3912	Sigma-Aldrich
Citric Acid	C2404	Sigma-Aldrich
Dimethyl sulfoxide	D8418	Sigma-Aldrich
Doxycycline hyclate	D9891	Sigma-Aldrich
EML425	5646	Tocris Bioscience
Esha 2000	79051	eSHa Aquarium products
Ethanol	64-17-15	VWR International
Ethylenediamineteraacetic acid disodium slat dihydrate, EDTA	E6635	Sigma- Aldrich
5-Ethylnyl-2'-deoxyuridine (EdU)	A10044	Sigma-Aldrich
Gel loading dye,6X,no SDS	B7025S	NEB
Glycerol	G5516	Sigma-Aldrich

Glycine	G8898	Sigma-Aldrich
Histoacryl Tissue seal	9381104	Braun
Hydrochloric acid, HCl	320331	Sigma-Aldrich
Kanamycin	BPE906-5	Fisher Scientific
Laemmli Sample Buffer	1610747	BioRad
Lipopolysaccharide (LPS)	L2880	Sigma-Aldrich
Methanol	67-56-1	Fischer Chemicals
Methylene Blue	370.0025	VWR International
Mocetinostat	18287	Cayman Chemicals
Needles, 0.3x13mm	304000	BD Microlance 3
Normal donkey serum	S30	Millipore
PageRuler Plus Prestained Protein Ladder	26619	ThermoScienctific
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich
Ponceau S solution	P7170	Sigma-Aldrich
Potassium chloride, KCl	P9541	Sigma-Aldrich
Potassium dihydrogen orthophosphate, KH_2PO_4	CHE2948	Scientific Laboratory Supplies
Protease Inhibitor Cocktail Tablet	04693159001	Roche
Pomalidomide	19877	Cayman Chemicals
Quickload 100bp DNA ladder	N0468S	NEB
Quickload 1Kb DNA ladder	N0551S	NEB
Sodium chloride, NaCl	S7653	Sigma-Aldrich
Sodium phosphate dibasic, Na_2HPO_4	S0876	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	BP166	Fisher BioReagents
Sodium hydroxide, NaOH	30620	Sigma-Aldrich
Superfrost coated glass slides	631-0108	VWR International
Superpremium microscope slides	631-0116	VWR International
Syringes,1ml	SS+01T1	Terumo
N,N,N',N'-Tetramethylethylenediamine (TEMED)	T9281	Sigma-Aldrich
10X TGS Buffer	1610732	BioRad
Triton X-100	327372500	Acros Organics
Trichostatin A	T8552	Sigma-Aldrich

Trizma Base	93362	Sigma-Aldrich
Tween 20	P1379	Sigma-Aldrich

2.5.4 Kits

Name	**Cat No.**	**Source**
Click-iT EdU Alexa Flour 647 Imaging Kit	C10340	Invitrogen
iScript cDNA synthesis Kit	1708890	BioRad
QiagenPlasmid PlusMidi Kit	12943	Qiagen
Qiaprep spin Miniprep Kit	27106	Qiagen
QIAquick PCR purification Kit	28106	Qiagen
QIAquick Gel Extraction Kit	28706	Qiagen
Pierce BCA Protein Assay Kit	23227	ThermoScientific
RNeasy Mini Kit	74106	Qiagen

2.5.5 Solutions

Blocking buffer (wholemount immunohistochemistry)	1X PBS 1%DMSO 1%BSA 1% Normal Donkey serum 0.7% TritonX-100
Paraformaldehyde 4%	16g Paraformaldehyde 40ml 10X PBS complete with dH_2O up to 400ml
10X Phosphate buffered saline (PBS)	160g NaCl 28.3g Na_2HPO_4 4g KCl 4.8g KH_2PO_4 Complete with dH_2O up to 2L
Citric Acid ,10mM	0.960g Citric Acid Complete with dH_2O up to 500ml adjust pH to 6 with NaOH
50X TAE buffer	484g TrisBase 114.2ml Acetic Acid 1000ml 0.1 EDTA,pH8 Adjust pH to 7.5 with HCl Complete with dH_2O up to 2L
Running Buffer	100ml 10X TGS Buffer Complete up to 1L with dH_2O
Transfer Buffer	100ml 10X TGS Buffer 200ml Methanol Complete up to 1L with dH_2O
Tris Buffer, 0.5M	6.06g Trizma Base Adjust pH to 6.8 with HCl Complete with dH_2O up to100ml

Tris Buffer, 1.5M	18.18g Trizma Base Adjust pH to 8.8 with HCl Complete with dH_2O up to 100ml

Chapter 3 Generation of a system to manipulate Hdac1 levels in zebrafish

3.1 Introduction

To determine the role of Hdac1 in neuroregeneration a genetic system that enables the manipulation of Hdac1 levels was generated. This was to address two problems with pharmacological methods used so far. Firstly, HDAC inhibitors target every cell in the zebrafish. They do not provide evidence for which cell type the Hdac1 is acting in that subsequently leads to changes in neuroregeneration. This is particularly important in the context of injury whereby an immune response is elicited. Hdac1 has well documented roles in immune cell activation (Hull, Montgomery and Leyva, 2016) and many HDAC inhibitors lead to immune suppression e.g. they reduce proinflammatory cytokine production (Leoni *et al.*, 2002; Di Liddo *et al.*, 2016). Previous work from our group has found that an immune response is necessary for neuroregeneration after spinal cord injury in larval zebrafish (Ohnmacht *et al.*, 2016). Therefore, a concern with using the HDAC inhibitors is that the effect observed could be secondary to the immune response being suppressed rather than an intrinsic change occurring in the ERGs. To fully understand if Hdac1 activity in the ERGs is necessary for neuroregeneration a cell specific loss of function system is required. I generated a transgenic line to express a dominant negative form of the enzyme (dnHdac1) selectively in the spinal cord ERGs.

Secondly, there is no pharmacological compounds that can activate Hdac1. There is an abundance of compounds with Hdac inhibition properties but none available that can increase Hdac activity. Indirectly, acetylation can be altered by blocking the enzymes that add on the acetyl groups; Histone acetyltransferases (HATs), but no compounds can act directly on Hdac1. HAT inhibition will lead to a decrease in acetylation but may not lead to the same changes in gene expression as Hdac1 activity. Therefore, to facilitate gain of function experiments a genetic mechanism by which Hdac1 can be overexpressed selectively in spinal cord ERGs was generated.

Hdac1 zebrafish mutants have been generated previously to study the function of Hdac1 during development (Cunliffe, 2004; Stadler *et al.*, 2005; Yamaguchi *et al.*, 2005). They are embryonic lethal. They display severe phenotypes in many tissues including the CNS. Therefore, a genetic manipulation that would be temporally and spatially controlled was required to avoid any consequences to regeneration that stemmed from development problems or an altered immune response. There are

various techniques available in zebrafish that could be used to express dnHdac1 and/or Hdac1.

3.2 Overexpression methodologies

3.2.1 Cre-Lox

This system takes advantage of the Cre recombinase protein's ability to cut certain DNA sequences, called loxP sites, within the genome (Sternberg and Hamilton, 1981). LoxP sites can be positioned around the gene of interest. Cre activity will cause the removal of the genomic region located between the loxP sites, generating a knockout. The selective expression or modifications to the Cre protein provides the system with spatial and temporal control. Specific promotor elements can drive the expression of Cre protein in a tissue of interest to avoid global effects (Akagi *et al.*, 1997). Temporal control can be achieved by driving the expression of the Cre protein under a heat shock promoter (Le *et al.*, 2007) or by fusing the ligand binding domain of the human estrogen receptor to the Cre protein (CreERT2). CreERT2 will remain in the cytosol (i.e. inactive) until the addition of the drug tamoxifen whereby it enters the nucleus allowing genome editing to occur (Feil *et al.*, 1996). Importantly, the changes in the genome caused by Cre-Lox activity are irreversible. This makes it useful for applications such as lineage tracing as the progeny of the targeted cells will permanently express the genetic label. In this study, however, Hdac1 level manipulations limited to within the spinal cord ERGs, and not in their subsequent progeny, was desired.

3.2.2 Heat shock

Heat shock protein promoter can be used to regulate gene expression. This promoter induces downstream gene expression at higher ambient temperatures, but are silenced at normal temperatures. In zebrafish the most common heat shock protein promoter is the 1.5kb hsp70l promoter (Halloran *et al.*, 2000; Shoji and Sato-Maeda, 2008). Raising the temperature of the water from 28.5℃ to 38℃ for 30minutes is sufficient to drive the target gene expression. This system though enables temporal control is often not linked to spatial control. The characteristics of the specific transgenic line determine whether the cells of interest are targeted. Some studies (Dias *et al.*, 2012; Kizil, Dudczig, *et al.*, 2012) achieved relative specific expression of their constructs to progenitors in the spinal cord which was advantageous for their studies, but was not achieved through selective targeting of these cells.

3.2.3 GAL4-UAS

The GAL4-UAS system is a two-component transcriptional activation switch originally from *Saccharomyces cerevisiae*. Primarily used in *Drosophila* (Brand and Perrimon, 1993), this system has also been applied to mice and zebrafish. The Gal4 transcription factor binds to a unique DNA sequence, called Upstream Activating Sequence (UAS). The Gal4-UAS system can be adapted for transgenesis studies in biological systems given that any gene of interest can be inserted downstream of a UAS promoter cassette. Tissue specific promoters used to express Gal4 can determine the spatial domain of the UAS controlled transgene expression. Gal4 protein can be fused to the ligand binding domain of the estrogen receptor (Gal4-ERT) or regulated by heat shock promoter to provide temporal control of the transgene expression. This system has been developed for use in zebrafish (Scheer *et al.*, 2001; Gerety *et al.*, 2013; Akerberg, Stewart and Stankunas, 2014). The highly repetitive UAS sequences used in these transgenic lines often undergo transcriptional silencing (Akitake *et al.*, 2011) and therefore display limited adult inducibility. A Gal4-UAS transgenic line to label motor neurons previously used in the lab was not re-expressed in the regeneration context (Ohnmacht *et al.*, 2016) indicating this system may not always be suitable for regeneration studies.

3.2.4 CRISPR

CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 system has been used extensively to edit the genome for knock out studies in various biological systems. Specific guide RNA (gRNA) sequences that are complementary to regions within the gene of interest are used to target the nuclease protein, Cas9, to that genomic region. Cas9 activity will cause double stranded breaks in the DNA sequence. The endogenous DNA repair system in the cell often leads to improper repair and can lead to gene mutations. If these mutations are severe enough it can cause loss of function of the targeted gene. This technology has been altered to enable gene regulation. The Cas9 protein has been mutated at two points (D10A, H840A) to create a nuclease-deactivated form which cannot generate the double strand break. This dCas9, together with specific gRNAs, can still target the genomic regions and can be fused to effector domains resulting in transcriptional control of targeted regions. CRISPR-a (activation) uses dCas9 fused to activation domains such as VP160 and VP48. When combined with gRNAs that target the gene's promoter or transcriptional start site it can upregulate the expression of the gene of interest (Cheng *et al.*, 2013). CRISPR-I (inactivation) uses a dCas9 fused to KRAB (Krüppel

associated box) domains which with the gRNAs will target the gene for repression or knockdown (Qi *et al.*, 2013). The system can be made inducible with the addition of Cas9-ERT2 which allows for tamoxifen controlled activation (Liu *et al.*, 2016). However, strategies are still mainly in development to gain full spatial and temporal control of the system (Dai *et al.*, 2018).

Overall these strategies have the possibility that all cells in the zebrafish can be targeted which is not desirable when considering the function of the gene of interest may be different depending on the cell type; Hdac1 in the progenitor compared to in the neuron or the immune cell. A different system in which temporal and spatial control could be combined is the Tet-On system.

3.2.5 Tet-On system

Tetracycline controlled transcriptional activation is a method for inducing gene expression where transcription is turned on or off by the presence of tetracycline or one of its derivatives e.g. doxycycline (Gossen *et al.*, 1995). It takes advantage of mechanisms that gram-negative bacteria use to provide resistance to tetracycline antibiotics. It uses the tetracycline transactivator (tTA) protein which is a fusion of the tetracycline repressor (TetR) from *E. coli* and the activation domain of VP16 from herpes simplex virus. The tTA protein will bind to DNA at specific Tet operator sequences. This sequence has been combined with minimal promotors and called the Tet Responder element (TetRE). The TetRE will increase the gene expression downstream of themselves after the tTA protein binds. This system has been used in two manners. In Tet-Off, expression of TetRE controlled genes are repressed in the presence of tetracyclines. This is because the tetracycline binds to tTA protein rendering it incapable of binding to TetRE (top of Figure 3.1). The opposite occurs in Tet-On, as the tTA protein has been modified (called rtTA) so instead it will only bind to TetRE in the presence of tetracyclines (bottom of Figure 3.1).

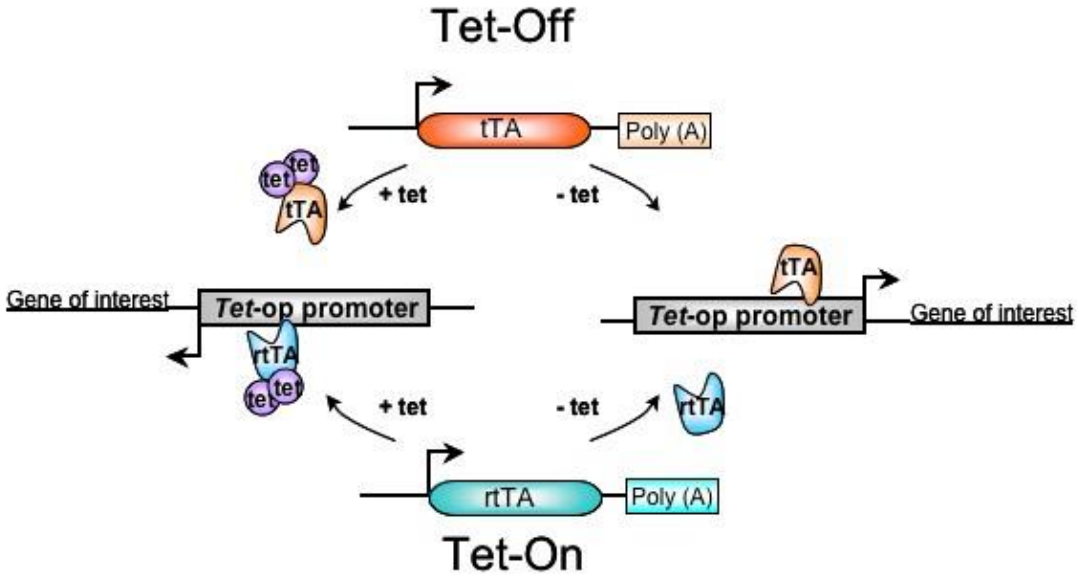


Figure 3-1: Cartoon of Tetracycline controlled transcriptional activation. The two different mechanisms Tet-Off (top) and Tet-On (bottom) are shown. In the presence of tetracyclines the Tet-Off system switches off gene expression while the Tet-On system switches gene expression on. Picture from https://www.genoway.com/technologies/tet/tet-system.htm

The Tet-On system has been used to drive gene expression in zebrafish tail fin (Wehner *et al.*, 2014) and photoreceptors in the retina (Campbell, Willoughby and Jensen, 2012; West *et al.*, 2014). For use in zebrafish the system relies on two transgenic lines. Firstly, an activator line in which tetracycline induced Tet activator (rtTA) is expressed under a tissue specific promoter. Secondly, a responder line in which the TetRE controls the expression of the gene of interest. The combination of the two transgenic lines generates animals in which the expression of the gene of interest is spatially and temporally controlled.

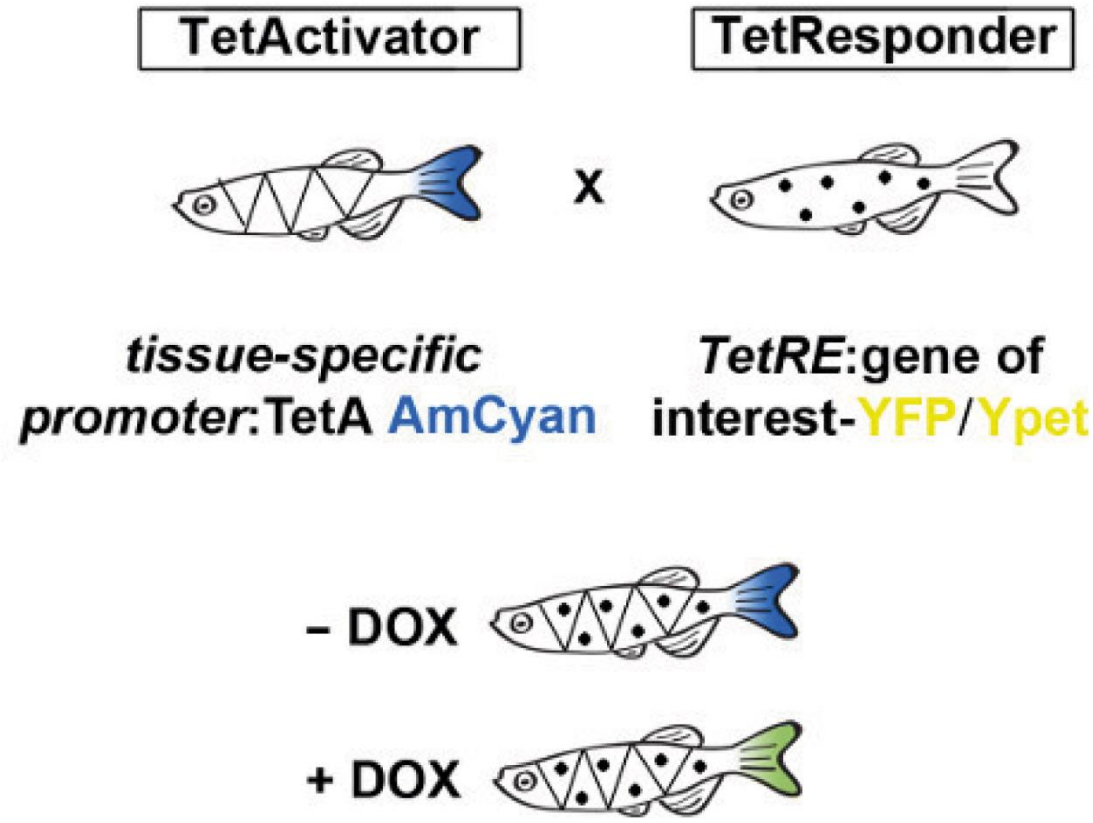


Figure 3-2: Cartoon showing strategy for achieving tissue specific inducible gene expression using the Tet-On system. Crosses between TetActivator and TetResponders produces animals in which the gene of interest can be induced with Doxycycline treatment (DOX).From (Wehner, Jahn and Weidinger, 2015) .

There are several considerations when using the Tet-On system. The expression of the TetRE regulated transgene may be influenced by its chromosomal insertion site. Therefore, it is important to select multiple founders to ensure optimal expression in the tissue of interest. Doxycycline is the most commonly used inducing agent as it is more stable than other tetracyclines (Gossen *et al.*, 1995). Doxycycline has been found to inhibit aspects of the immune system, such as macrophage polarisation (He and Marneros, 2014) and cytokine release (Cazalis *et al.*, 2008). However, this effect is seen at much higher concentrations (2-20µM) than the amount that is used for induction purposes (485nM). Putting all these considerations together the Tet-On system was chosen for the study as it was the best system for achieving both spatial and temporal control of Hdac1 levels.

3.2 Results

3.2.1 Generation of Transgenic lines

The conservation between the Hdac1 protein in humans, mice and zebrafish is very high as shown in Figure 3.3. Zebrafish Hdac1 is 93% similar to human HDAC1,

contains all the key domains of human HDAC1 and has the same sequence composition at the deacetylase active sites (Pinho *et al.*, 2015). A point mutation in the mammalian protein at position 141, which changes a histidine to an alanine, has been described as a dominant negative form of the protein. This mutation has been found in the mammalian protein to render the enzyme catalytically inactive yet maintain its ability to interact with binding partners (Hassig *et al.*, 1998; A Mal *et al.*, 2001). This mutant is commonly referred to as a dominant negative protein due to its ability to block Hdac1 mediated events such as; p53 deacetylation after UV damage (Ito *et al.*, 2002), prevent TGF-B induced apoptosis (Lei *et al.*, 2010) and Hdac1 dependent transcription (A. Mal *et al.*, 2001; Beharry *et al.*, 2014). The dominant negative form of the zebrafish protein, to the best of our knowledge, had not been constructed before or tested in an *in vivo* context. I cloned wild type Hdac1 from zebrafish cDNA and used site directed mutagenesis to change the histidine at amino acid position 142 to an alanine (marked by black arrow in Figure 3.3). Due to the addition of a leucine residue in the beginning of the zebrafish protein this is the equivalent position to the mammalian 141 position. Due to the high homology between the protein sequences it is likely that the H142A mutation in the zebrafish will generate a dominant negative protein.

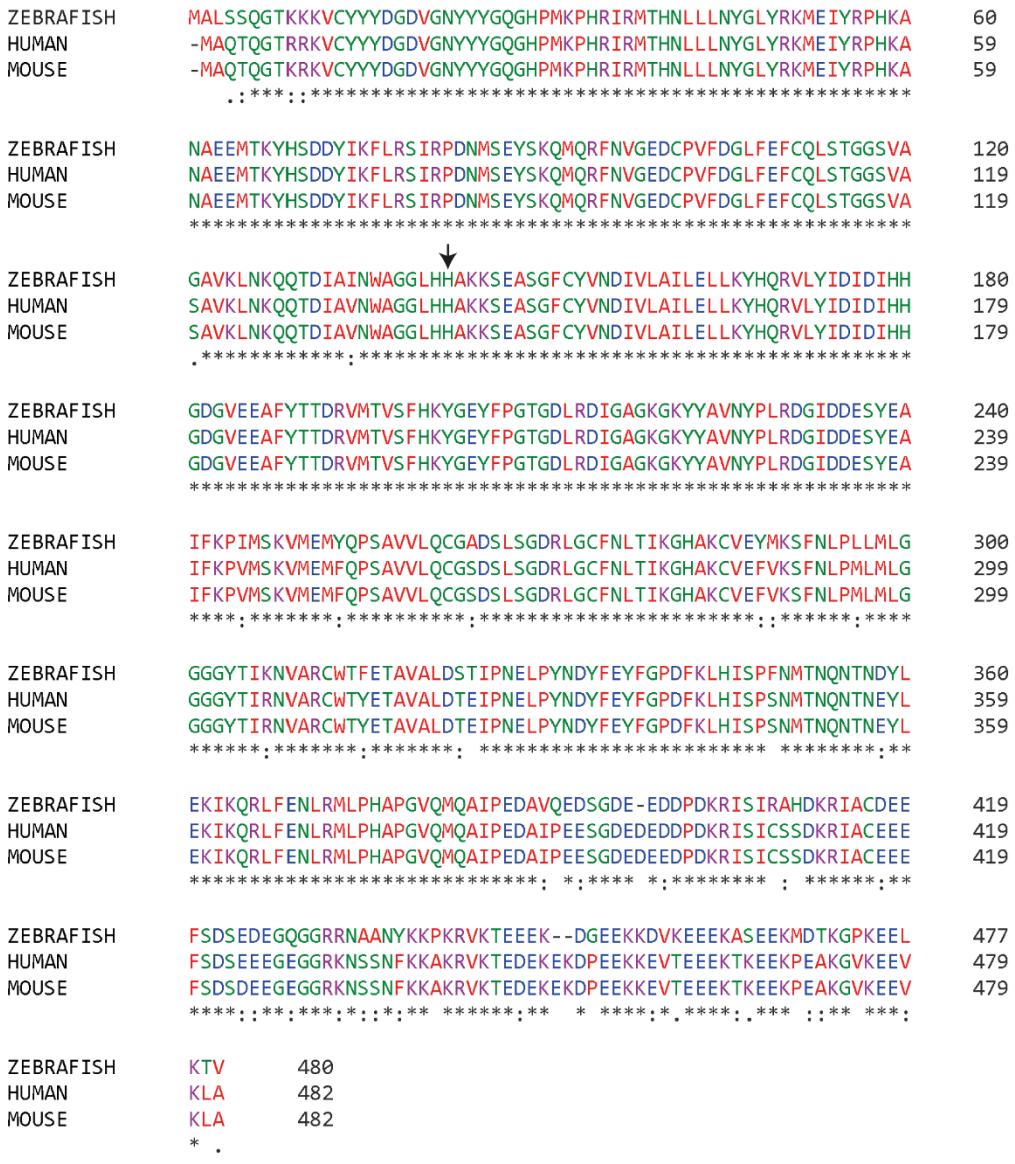


Figure 3-3: Comparison of zebrafish, human and mouse Hdac1 protein sequences. Alignment performed with Multiple Sequence Alignment Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) using protein sequences of zebrafish (NP_775343.1), mouse (NP_032254.1) and human (NP_004955.2). Colours of residues based on their physiochemical properties; red (small), blue (acidic), magenta (basic) and green (hydroxyl, sulfhydryl, amine, G). Symbols below indicate levels of consensus between the sequences; asterisk (*) indicates conserved residue, colon (:) indicates strongly similar- >0.5 in Gonnet PAM 250 matrix and period (.) indicates weakly similar- =<0.5. Black arrow points to histidine at position 142 in the zebrafish protein sequence.

To allow visualisation, I placed a yellow fluorescent protein (YFP) tag onto the N-terminus of both the wildtype Hdac1 and dnHdac1 proteins. Previous studies have not shown any negative effect on protein function when a fluorescent tag was added to this region of the protein (Tou, Liu and Shivdasani, 2004). I cloned the different

forms of Hdac1 into plasmids containing the TetRE. The two plasmids were injected into the single cell of wildtype zebrafish following Tol2 transgenesis procedures (Suster *et al.*, 2009).

Founder adult fish were then screened in two steps. Firstly, potential founder animals were crossed to a ubiquitous activator line, for ease of seeing expression, and the offspring screened for YFP expression after doxycycline treatment. Adults who generated YFP expressing embryos were then crossed to the ERG specific activator line *Her4.1*:irtTAM2(3F)-p2a-AmCyan (abbreviated in this study as *Her4.1*:TetA) to ensure correct expression in the cells of interest. The promoter sequence of this transgenic line has also been called *Her4.3* in previous studies (Wehner *et al.*, 2014). Adult fish whose offspring displayed strong specific expression were selected to establish the two transgenic lines, representative examples are shown in Figure 3.4B.

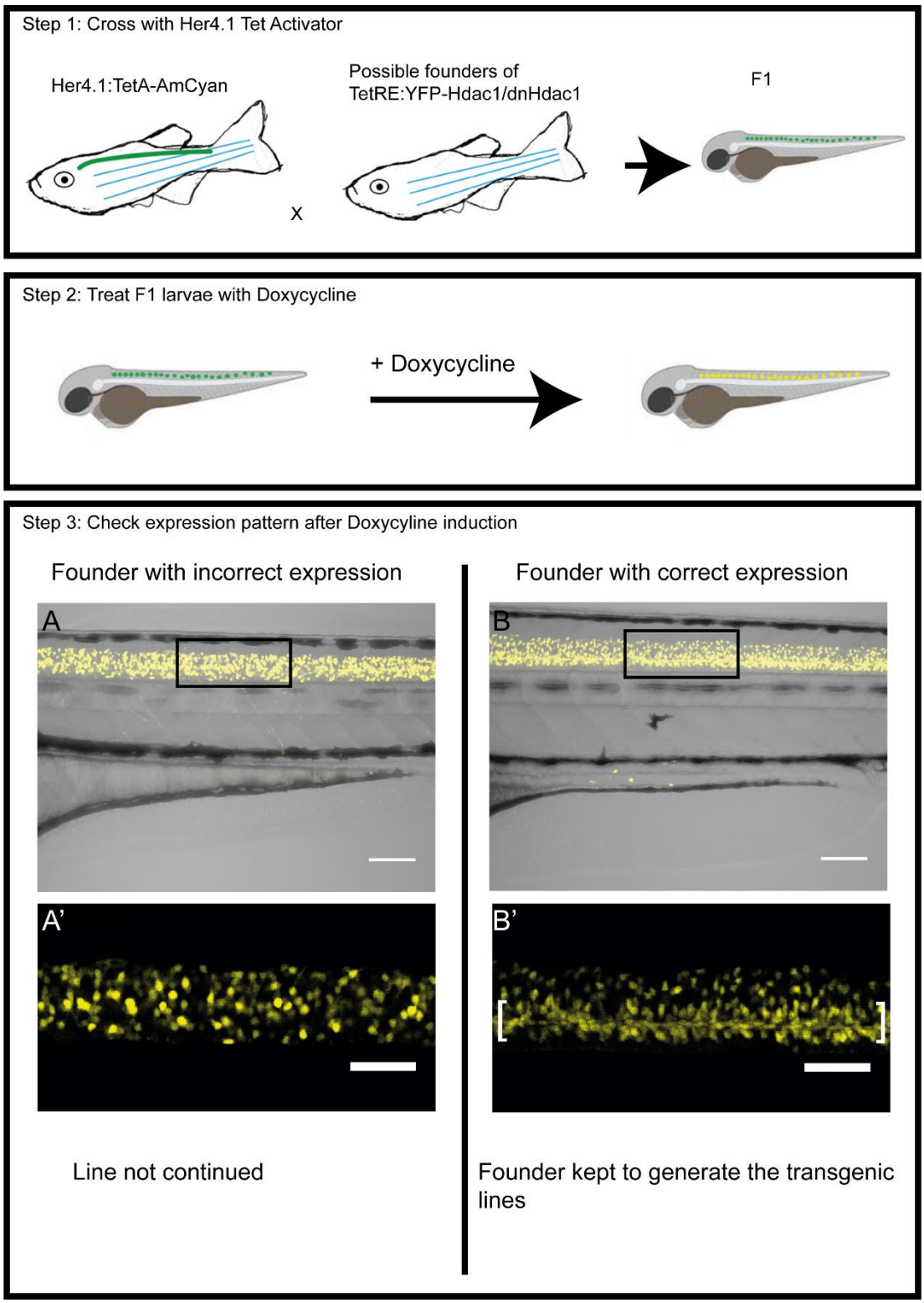


Figure 3-4: Breeding strategy for generation of the new TetResponder transgenic lines. Injected embryos with the TetResponder construct were raised to adulthood and bred with Her4.1 TetActivator fish to produce F1 larvae (step 1). F1 larvae were treated with doxycycline and screened for the appearance of YFP cells (step 2). Founders that produced offspring with correct homogenous expression were selected for line generation (step 3). **A-B**; Representative pictures of larval wholemounts after 24 hours of doxycycline treatment. Lateral

view is shown; rostral is left. Scale bar is 100µm. **A'-B'**; Close up of the spinal cord within the black boxes in A and B. White brackets in B' indicate correct expression in the progenitors. Scale bar is 10µm.

3.2.2 Transgenic lines show selective expression in the ependymo-radial glial cells

Her4.1 (hairy-related) is a notch target gene that is expressed throughout development in the ERGs of the zebrafish CNS. Cross sections of *Her4.1* reporter fish at 5dpf show that *Her4.1*:GFP positive cells can be observed in the middle of the spinal cord (Figure 3.5A). The GFP positive cells line the central canal demonstrating they label the spinal cord progenitor cells. Some of these co-label with *Olig2*:DsRed (white arrows in Figure 3.5A'-C) indicating the pMN domain progenitors which generate the motor neurons are *Her4.1* positive. Some additional cells in the spinal cord are also labelled in the *Her4.1*:GFP transgenic line, including secondary motor neurons (Yeo *et al.*, 2007). The additional cells labelled with *Olig2*:DsRed are motor neurons and oligodendrocytes. *Her4.1* expression increases after a lesion in the adult spinal cord (Dias *et al.*, 2012) and this regulatory sequence has been used for lineage tracing experiments of progenitors in the adult zebrafish brain after injury to the telencephalon (Kroehne *et al.*, 2011). The *Her4.1* Tet activator line was used in the zebrafish caudal fin (Wehner *et al.*, 2014) to drive expression in proliferating cells of the proximal medial blastema but has not been used before in studies in the spinal cord.

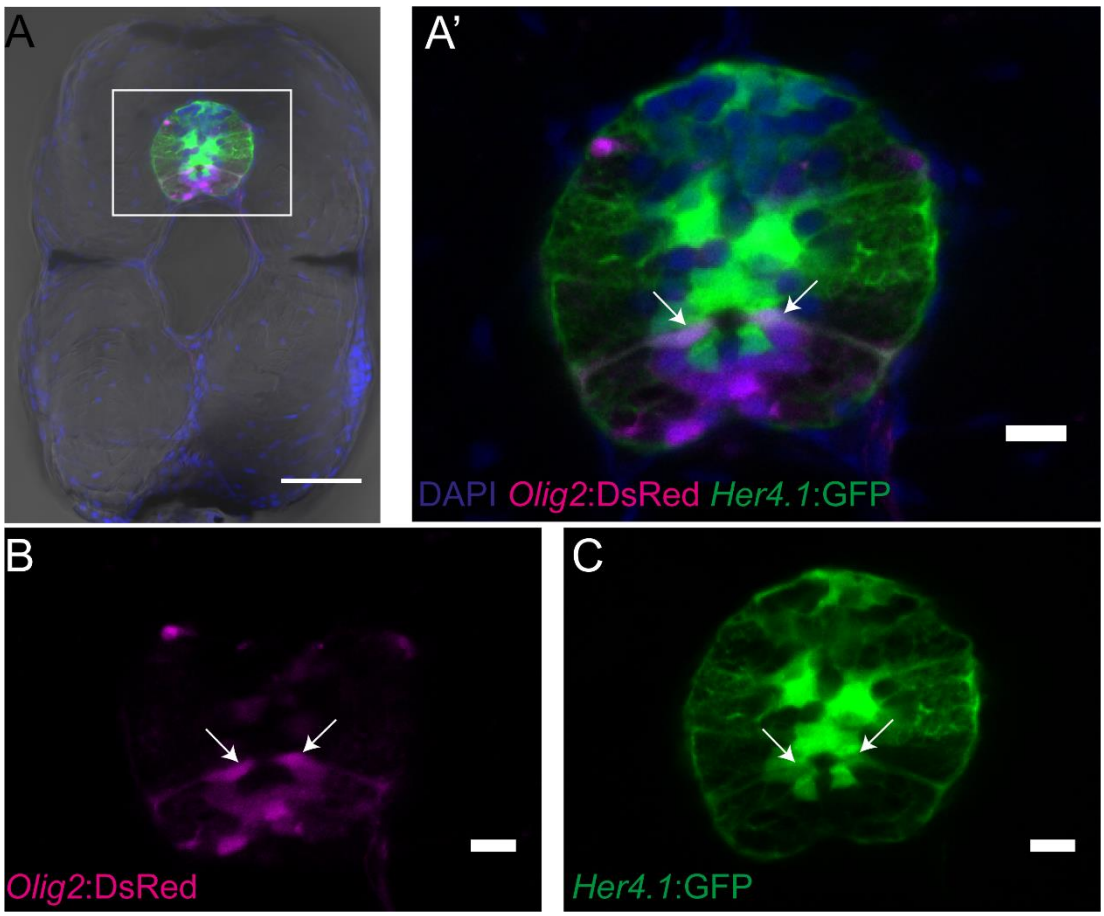


Figure 3-5: *Her4.1* labels the ERGs in the zebrafish spinal cord including the pMN domain. A; Cross section of 5dpf double transgenic *Olig2*:DsRed;*Her4.*1:GFP fish. Scale bar is 50µm. **A'**; Close up of the white box marked in A. White arrows point to double labelled cells which are pMN ERGs. **B-C**; Close up for A' in single channel for DsRed (B) and GFP (C). White arrows point to the double labelled cells. Scale bar A'-C is 10µm.

To confirm that the cells that were targeted by the *Her4.1* Tet activator line are the spinal cord ERGs I performed immunohistochemistry to label the progenitors and the neurons on cross sections of 4dpf larvae after 24hours of doxycycline treatment (Figure 3.6A). I used anti-glial fibrillary acidic protein (GFAP) to label the cytoskeleton of the progenitor cells all around the central canal and anti-HuC to label neuronal cells. The YFP expression is mainly in cells in the centre of the spinal cord, adjacent to the central canal. The YFP expression, which appears nuclear, is surrounded by GFAP staining. These double positive cells reside at the ventricle and displays the characteristic process that stretches out to the edges of the spinal cord (Figure 3.6B). The YFP expressing cells are not neurons as YFP expression does not colocalise with the HuC (Figure 3.6C, E, F). Together these confirm that the cells in the spinal cord targeted by the *Her4.1* Tet activator line are the ERGs.

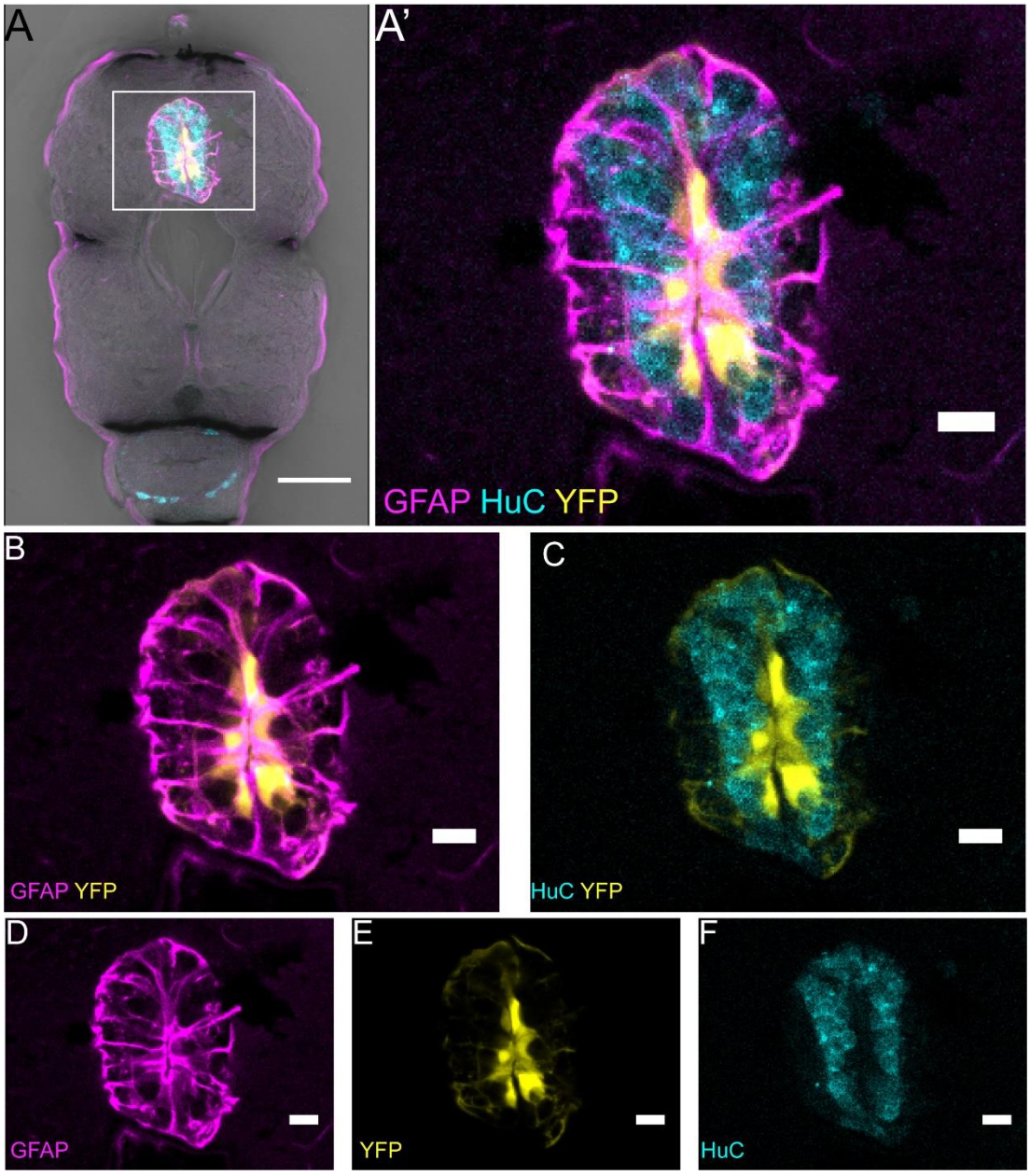


Figure 3-6: *Her4.1* Tet activator lines drives expression in the spinal cord progenitors. **A**: Section of 4dpf *Her4.1*:TetA;TetRE:YFP-Hdac1 (yellow) transgenic animal after doxycycline treatment labelled with anti-GFAP (magenta) and anti-HuC (cyan). Scale bar 50µm. **A'**: Close up from within the area marked out by white box in A. **B-C**; Close from A' with different combinations of two labels. **D-F**; Close from A' with single labels. Scale bar A'-C is 10µm.

3.2.3 Expression in the transgenic lines is induced by doxycycline treatment in larval and adult stages

A common consequence to genetic overexpression strategies is uninduced leaky expression of the transgene. To assess the level of uninduced expression of the new transgenic lines, double transgenic 3dpf larvae were treated with either vehicle or doxycycline. They were imaged for YFP expression after 24 hours. In the vehicle

treated group there was some faint expression in some animals (Figure 3.7A) however it was to a significantly lower level then what was observed after doxycycline treatment (Figure 3.7B). Therefore, in the larvae there is some leaky expression of the Hdac1 constructs, but it is almost minimal.

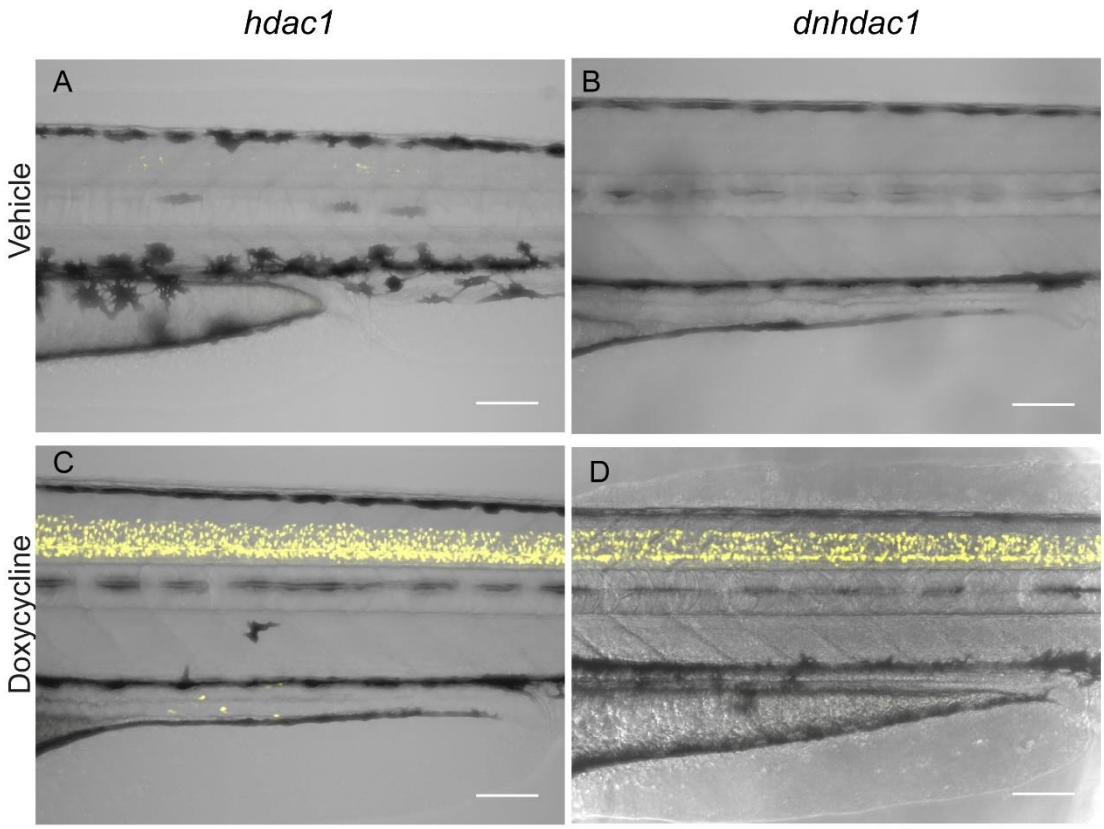


Figure 3-7: Doxycycline treatment leads to induction of transgenes in larval zebrafish. A-D; Representative pictures of wholemount *Her4*.1:TetA;TetRE:YFP-Hdac1 (A,C) or *Her4.1*:TetA;TetRE:YFP-dnHdac1 (B,D) 4dpf larvae after 24 hours of Vehicle (A,B) or Doxycycline (C,D) treatment. Lateral view shown; rostral is left. Scale bar is 100µm.

As it would be of interest to also use adult zebrafish to study regenerative neurogenesis, I tested whether the newly generated transgenic lines were able to be induced into adulthood (over 3months old). I found that, unlike the larval stages, doxycycline treatment in the double transgenic adult fish did not induce YFP expression in the unlesioned spinal cord (Figure 3.8a,b). *Her4.1* expression is upregulated after a lesion (Dias *et al.*, 2012) and when doxycycline was applied to lesioned double transgenic *Her4.1*:TetA:TetRE:YFP-Hdac1 animals YFP expression was observed all around the central canal (Figure 3.8c,d). The YFP expressing cells

appeared in same location as the *her4.1* lesion induced expression seen previously (Dias *et al.*, 2012). When I quantified the numbers of YFP positive cells I found a rostral to caudal asymmetry. This is similar to what is found with the numbers of new born motor neurons after a lesion (Reimer *et al.*, 2013; Barreiro-Iglesias *et al.*, 2015). In the *Her4.1*:TetA;TetRE:YFP-Hdac1 animals there was 805±170.5 YFP positive cells in the spinal cord rostral to the lesion and 313±112.3 YFP positive cells in the spinal cord caudal to the lesion (Figure 3.8B). Adult inducibility was not consistent across the lines generated, however. When *Her4.1*:TetA;TetRE:dnHdac1 double transgenic animals were lesioned and treated with doxycycline there was significantly reduced numbers of YFP positive cells compared to the wildtype line, 221.5±70.54 cells in the rostral spinal cord and 70.4±28.62 cells in the caudal spinal cord (Figure 3.8B) . Double transgenic fish that were lesioned and treated with vehicle had no YFP positive cells (Figure 3.8e) showing expression of the transgenes can be controlled by doxycycline treatment.

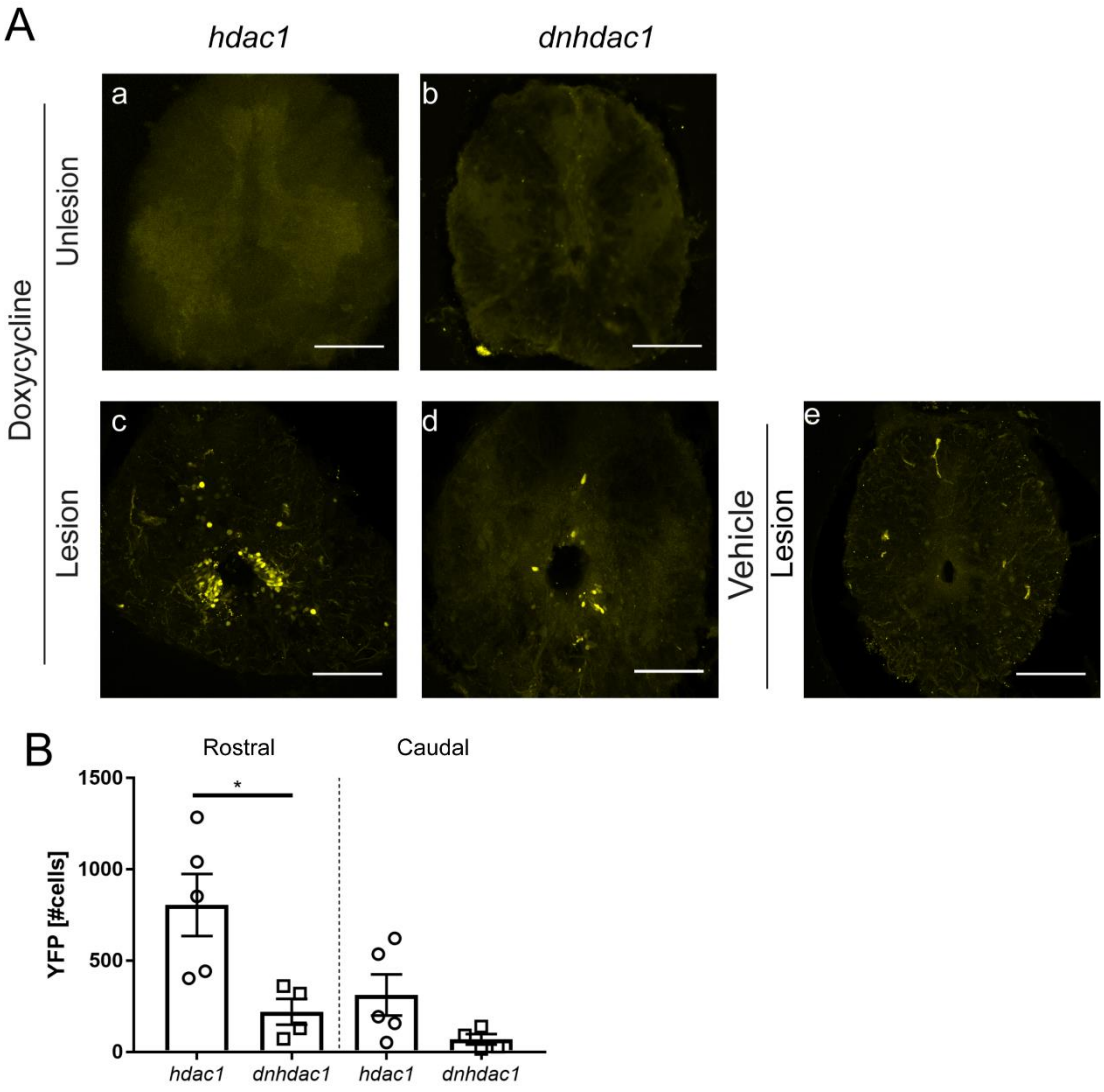


Figure 3-8: Induction of transgene in adult zebrafish after injury. **A**: Representative images of expression of YFP-Hdac1/YFP-dnHdac1 (yellow) labelled with anti-GFP in cross sections of the adult spinal cord after doxycycline (a-d) or vehicle (e) treatment in lesioned (c-e) or unlesioned (a-b) in *Her4.1*:TetA; TetRE:YFP-Hdac1 (a, c) or *Her4.1*:TetA;TetRE:YFP-dnHdac1 (b, d) double transgenic animals. Dorsal is up. Scale bar is 100µm. **B**: Quantification of the number of YFP positive cells in the adult spinal cord 14 days after injury. Double transgenic *Her4.1*:TetA; TetRE:YFP-Hdac1 (circles) or *Her4.1*:TetA;TetRE:YFP-dnHdac1 (squares) animals were treated with doxycycline after lesion. Numbers are separated according to location to the lesion site; rostral and caudal. Data shown as mean±SEM as a bar and scatter plot. Each dot is a fish, N=5 (*hdac1*) and 4(*dnhdac*1). Mann-Whitney test; rostral *P=0.0159, caudal P=0.0635.

3.2.4 Time-Course of Doxycycline-Induced Gene Expression

Larval regeneration occurs rapidly; new born motor neurons are observed by 48 hours after injury (Ohnmacht *et al.*, 2016). I next investigated how long it took for detectable levels of the Hdac1 constructs to be observed after doxycycline treatment to ensure

it occurred within the time frame of larval regeneration. Double transgenic larvae were induced at 3dpf to mirror the time frame of induction after a lesion. Larvae were fixed at different time points after doxycycline treatment (2,4,6,8,10 and 24hours) and put through anti-GFP immunohistochemistry to detect the YFP expression (Figure 3.9A-F). The number of YFP positive cells was analysed in three somite-segments at the end of the yolk extension. YFP positive cells could be observed from 2 hours post induction and their number increased over time. The numbers of YFP positive cells did not significantly change between 4 and 24 hours post induction (Figure 3.9G).

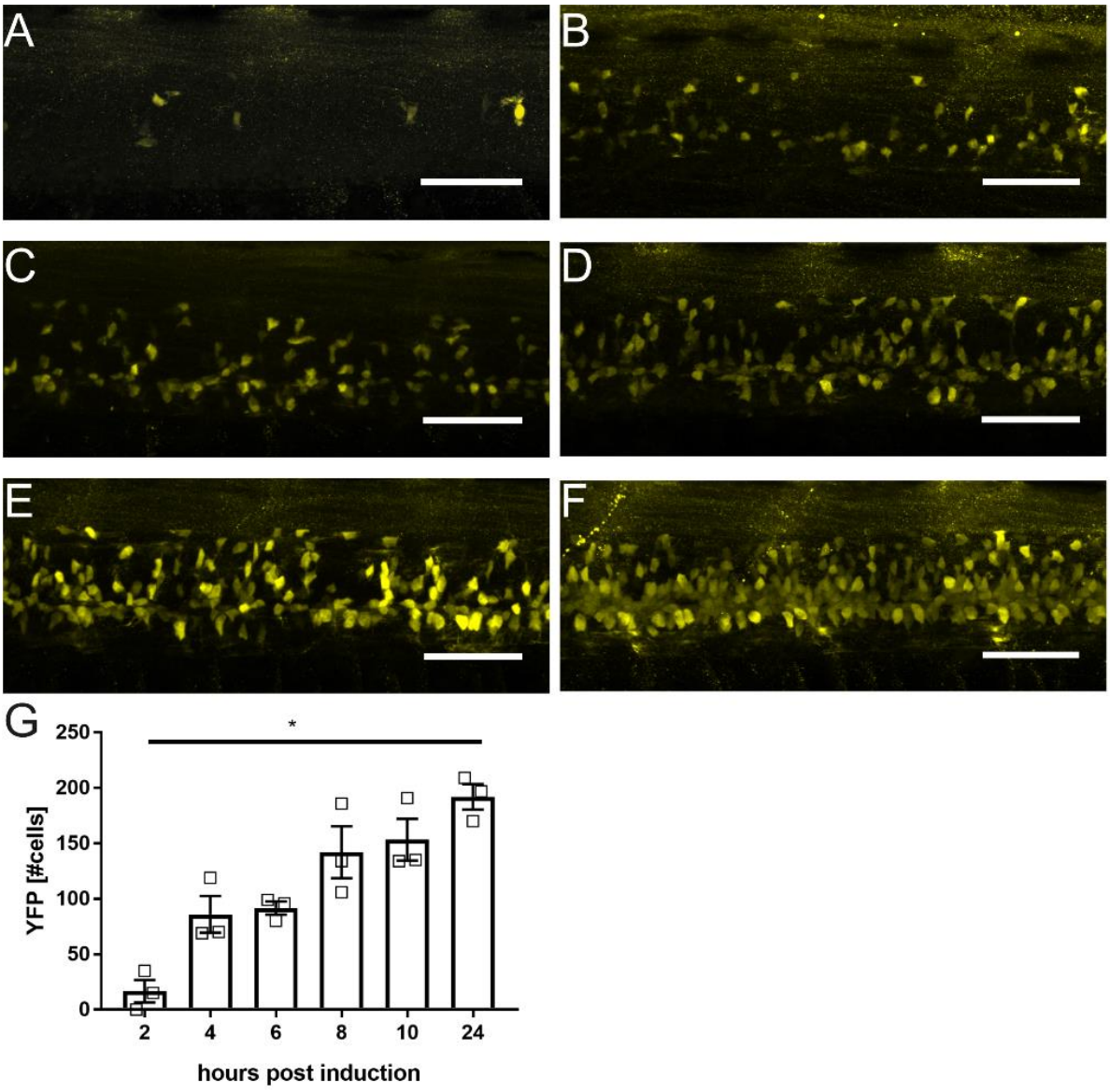


Figure 3.9: Induction of the transgene can be observed from 2 hours after doxycycline treatment. **A-F**: Representative images of YFP-Hdac1 in *Her4.1*:TetA;TetRE:YFP-Hdac1 double transgenic larval zebrafish at different hour timepoints after induction; 2(A), 4(B), 6(C),8(D),10(E), 24(F). Scale bar is 50µm. Rostral is left. **G**: Quantification of the number of

YFP positive cells within 3 somite segments at different time points post doxycycline induction. Data shown as mean±SEM, as a bar and scatter plot. Each dot is a fish. Krustal-Wallis with Duns multiple comparison test, *P=0.0150.

3.2.5 Transgenic lines increase Hdac1 expression

After an injury to the larval spinal cord Hdac1 expression around the lesion site was found to increase by 50% (unpublished observations). To observe the magnitude of overexpression that the new transgenic lines could achieve, I investigated the Hdac1 expression levels in the new transgenic line expressing wildtype Hdac1 after doxycycline induction. I used both the *Ubiquitin* activator line to express YFP-Hdac1 in all the cells of the fish and the *Her4.1* activator line to express YFP-Hdac1/YFP-dnHdac1 in the ERGs. I induced with doxycycline between 3-4dpf to mimic the lesion paradigm time scheme. RNA was extracted from the trunk of animals, after the head and tails had been removed, to copy the tissue region of the lesion site. This RNA was used in quantitative PCR to observe the expression of Hdac1 in double transgenic animals compared to wildtype controls. β-actin was used as the housekeeping control. After ubiquitous expression in the *Ubiquitin*:TetA;TetRE:YFP-Hdac1 double transgenic animals there is a 18 fold increase in Hdac1 expression levels (Figure 3.10A). After ERG specific expression there is a 3.5 fold increase in Hdac1 expression levels in the *Her4.1*:TetA;TetRE;YFP-Hdac1 double transgenic animals. While the *Her4.1*:TetA;TetRE:YFP-dnHdac1 double transgenic animals showed 2.5 fold increase in Hdac1 expression levels (Figure 3.10B). The lower fold increase in the ERG specific group is most likely due to the sample containing a small population of cells within the entire sample that are overexpressing Hdac1.

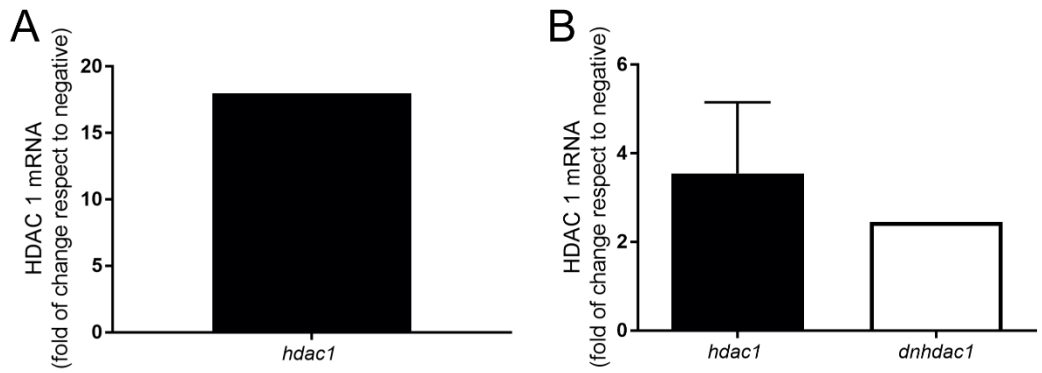


Figure 3.10: Tet-On system can increase Hdac1 expression levels. A; Quantification of Hdac1 expression in *Ubiquitin*:TetA;TetRE:YFP-Hdac1. **B**; Quantification of Hdac1 expression in *Her4.1*:TetA;TetRE:YFP-Hdac1 (black bar) or *Her4.1*:TetA;TetRE:YFP-dnHdac1 (white bar) double transgenic larval zebrafish normalised to wildtype controls 24 hours after doxycycline induction. β-actin was included as a housekeeping control. Data from 1 (A, B white bar) and 2(B black bar) independent experiments.

3.2.6 Preliminary investigation of acetylation levels

Similarly, the change in Hdac1 expression levels does not confirm the functionality of the constructs. The dnHdac1 construct should instead affect the function of the endogenous protein not its expression. To address this and confirm that the YFP tagged constructs were functioning by manipulating HDAC activity, the level of histone acetylation was examined in larval zebrafish after the expression of Hdac1 or dnHdac1. The levels of acetylated H4 protein was compared by western blot analysis. The ubiquitin activator line was used to drive the expression of constructs in all cells, to obtain sufficient protein for analysis (as seen in Figure 3.11A). Larvae were incubated in doxycycline from 3-4dpf and homogenised to extract protein. Total protein concentrations were assessed with a BCA assay to allow for equal protein loading for gel electrophoresis. The levels of acetylated H4 protein was compared across the groups and normalised to levels of the housekeeping protein alpha-tubulin. No obvious changes in acetylation could be observed after *hdac1* or *dnhdac1* expression.

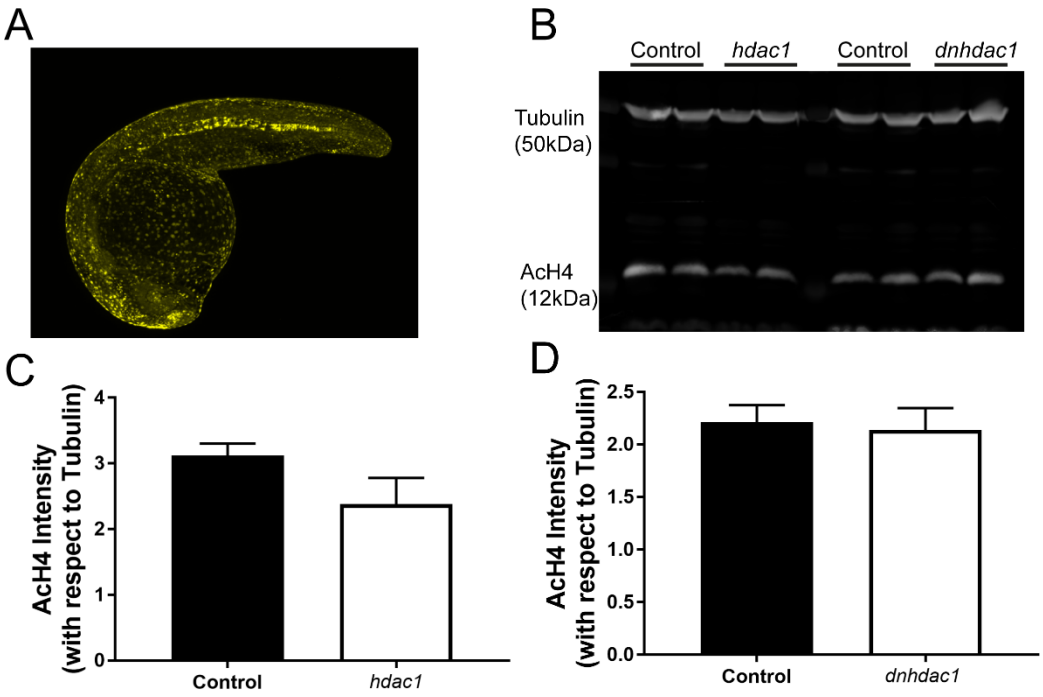


Figure 3.11: Preliminary investigation on acetylation levels after expression of *hdac1* or *dnhdac1*. A: Representative image of 24hpf *Ubiquitin*:TetA:TetRE:YFP-Hdac1 after induction. **B**: Western blot analysis of acetylated H4 (AcH4) on protein extracts from control, *hdac1* or *dnhdac1* expressing larvae. Samples were ran in duplicate. Tubulin was included as a control. **C-D**: Graphs of the intensity of the AcH4 signal after *hdac1* (C) and *dnhdac1* (D) expression normalised to intensity of tubulin signal. 1 independent experiment.

3.3 Discussion

In this chapter, I characterised the two transgenic lines that were generated to enable the conditional and cell specific manipulation of Hdac1 levels within the zebrafish spinal cord progenitor cells. The Tet-On system has multiple advantages over other expression systems to address the aims of the investigation.

3.3.1 Her4.1 Activator line drives expression in the ERGs

HDAC inhibitors are pleiotropic and as such may influence transcriptomic changes related to regenerative neurogenesis directly or indirectly. Hence, a cell specific manipulation was required to fully understand the role of Hdac1 in neuroregeneration. The *Her4.1* transgene is expressed in ERGs in the developing (Yeo *et al.*, 2007) and adult zebrafish spinal cord after injury (Dias *et al.*, 2012). The pMN ERG population is also labelled with this transgene. The *Her4.1* activator line that was generated previously (Wehner *et al.*, 2014) was confirmed to be capable of driving the expression of the Hdac1 forms in a population of cells of the spinal cord. The cells targeted are positive for the radial glia marker GFAP and negative for the neuronal marker HuC demonstrating they are predominately ERGs. There was some additional cells in the spinal cord targeted which may be the subset of secondary motor neurons that are labelled in the *her4.1* transgenic lines (Yeo *et al.*, 2007). Other ERG specific promoters could be tested for improved specific expression. *Her4.1* is not expressed to a sufficient degree in the adult spinal cord to enable experiments in the unlesioned spinal cord. A promoter that is expressed into adulthood could allow for such experiments such as; Sox2, Foxj1a and GFAP. Sox 2 regulatory elements have been used in mice to label adult progenitor cells in the spinal cord (Kang and Hébert, 2012). No sox2 reporter lines have been generated in zebrafish so far. Foxj1a is expressed by adult zebrafish ERGs in the unlesioned spinal cord (Ribeiro *et al.*, 2017) and a reporter line using a 0.6kb fragment upstream of the *foxj1a* gene has been generated which labels the ERGs (Caron, Xu and Lin, 2012). GFAP regulatory elements have been used to generate reporter transgenic zebrafish (Bernardos and Raymond, 2006) and expression can be observed in adult ERGs in the unlesioned spinal cord. The pMN domain (as measured by *Olig2*:DsRed expression), however, is not labelled with this reporter (Becker and Becker, 2015). Therefore, GFAP can be used for targeting expression in the other ERG populations but would not be suitable for investigating motor neuron regeneration.

3.3.2 Tet-On system provides temporal control to expression

To study the processes of regeneration a system that has no deficits in development is necessary. Therefore, to achieve this, a conditional manipulation of Hdac1 was required. The transgenic lines show expression mainly after induction with doxycycline. This avoids the developmental effects of inhibiting Hdac1 in the progenitors and enables the study of the role of Hdac1 specifically after injury. Hdac1 is ubiquitously expressed in early zebrafish embryos (de Ruijter *et al.*, 2003; Cunliffe, 2004) and different zebrafish mutants such as *hi*^{1618}*,add* and *colgate* display multiple deficits in organogenesis. Studies using *hdac1* mutants and morphants show that Hdac1 is required for differentiation of the retina (Stadler *et al.*, 2005; Yamaguchi *et al.*, 2005), melanophores (Ignatius *et al.*, 2008), inner ear (He *et al.*, 2016), motor neurons (Cunliffe, 2004), oligodendrocytes(Cunliffe and Casaccia-Bonnefil, 2006) and craniofacial cartilage (Pillai *et al.*, 2004). The phenotype of the *hi*^{1618} mutant is strong enough that mutants can be distinguished from wildtype siblings at 24hpf by their reduced anterior hindbrain development (Cunliffe, 2004). There was some expression of the tagged Hdac1 forms observed in vehicle treated double transgenic larvae. During development this *hdac1* or *dnhdac1* leaky expression could impact the development of the ERGs or the motor neurons. The uninduced double transgenic larvae, however, do not show any gross morphology alterations like the *hdac1* mutants. Therefore, it can be assumed that the leaky expression does not attain high enough expression to alter development. In support of this the leaky expression that is observed is significantly less in comparison to the expression that is achieved after induction with doxycycline. The transgenic lines did not display the same leaky expression throughout development, as vehicle treated adult double transgenics did not have any detectable YFP cells.

3.3.3 Tet-On system can be induced in adulthood

Expression of the transgenes in the *Her4.1* activator line is possible in the adult spinal cord after injury. The lack of induction in the unlesioned adult spinal cord could be due to the limited expression of *her4.1* (Dias *et al.*, 2012). This is supported by the ability of the transgenes to be induced after a lesion, which increases the expression of *her4.1*. Doxycycline in mammals can pass the blood-brain-barrier but at relatively low concentrations (Michel, Mosser and Olle, 1984; Karlsson *et al.*, 1996). Another tetracycline, minocycline may be more useful for CNS applications of the Tet-On system as its pharmacodynamics results in better tissue penetration (Saivin and Houin, 1988). Further investigations using the *ubiquitin* activator line could be

performed to ensure the lack of inducibility in the unlesioned spinal cord is not due to doxycycline's ability to access the CNS in adult zebrafish. Inducibility in adulthood is not always possible due to silencing of certain regions of the genome. Therefore, careful selection of founder animals that generate appropriate inducibility is important. The adult expression in the *Her4.1*:TetA;TetRE:YFP-Hdac1 double transgenic adults is variable but shows robust expression around the central canal. The *Her4.1*:TetA;TetRE:YFP-dnHdac1 double transgenic adults does not show as robust expression, as shown in example pictures in Figure 3.8. Therefore, additional founders will be screened to see if better adult inducibility is possible for this line.

3.3.4 Tet-On system induces expression of the transgene after 2 hours in the larval zebrafish

Doxycycline treatment induces expression rapidly in the larval zebrafish, with YFP positive cells observed in the spinal cord from 2 hours post induction. This time-course is similar to that found in other studies where doxycycline induced gene expression was observed after 2 or 4 hours in the retina (Campbell, Willoughby and Jensen, 2012; West *et al.*, 2014). This demonstrates that these transgenic lines can be used within the time frame of regeneration. Hdac1 levels significantly increase at 24 hours post injury in larval zebrafish. Therefore, the expression of the transgenes is in place to either reduce (dnHdac1) or increase (wildtype Hdac1) the activity of endogenous Hdac1. A time-course of induction in adult zebrafish would need to be performed to assess whether the induction dynamics is the same in adulthood.

3.3.5 The new transgenic lines change Hdac1 expression

The expression of wildtype *hdac1* using the Tet-On system increased the expression level of Hdac1 in the cell. The expression achieved by the ERG-specific transgenic line *Her4.1*:TetA;TetRE:YFP-Hdac1 increased the expression of Hdac1 by 350%. The primers used in this study amplified a fragment within the Hdac1 sequence and would recognise both the wildtype and dominant negative form. Therefore they could be used in combination with the *Her4.1*:TetA;TetRE:YFP-dnHdac1 to observe if induction of the construct was similar between the two transgenic lines. The expression of *dnhdac1* in the ERGs increased the total levels of Hdac1 (250%). The increase in expression in both transgenic lines is greater than the increase in expression that is induced by the lesion (350% or 250% compared to 50%). The expression levels achieved by the system in both transgenic lines should therefore be sufficient to exert a biological effect. The expression levels may be too high above the physiological levels, however, that they cause detrimental effects. This study did not test whether

the dose of doxycycline could lead to different levels of Hdac1 expression. This would give the system more flexibility and avoid possible toxic side effects of Hdac1 expression.

3.3.6 More experiments are required to confirm the new transgenic lines change acetylation

The western blot analysis of this study did not confirm that the expression of either *hdac1* or *dnhdac1* in the new transgenic lines altered acetylation levels in the cells. Additional experiments are required to enable the changes in acetylation to be statistically analysed. Additional antibodies that recognise acetylation modifications (Harrison *et al.*, 2011; Xiao *et al.*, 2018) could be tested. Immunohistochemistry is possible with some of the antibodies available, which combined with the ERG specific expression of the transgenes would provide additional evidence that acetylation is changed. Therefore, whether the H142A mutation that was directed in the zebrafish protein acts as a dominant negative is still to be determined.

3.3.7 Additional induction systems

The Tet-On system was useful for this study but future work could take advantage of improvements in the CRISPRa/CRISPRi system that increases its spatial control. The expression of the *dnhdac1* did not show the increase in acetylation levels as was expected. Directly preventing the transcription of Hdac1 with CRISPRi could be a more efficient method to inhibiting Hdac1 activity than the expression of a dominant negative protein. A combinatorial inducible promotor system that involves combination with the Tet-On system or the steroid inducing system would retain the inducibility with the effectiveness of CRISPR-Cas9 targeting. Doxycycline induces the expression of Cas9/dCas9 which is placed under the control of the TetRE. The Cas9 is guided to the desired genomic locus by the gRNAs. This system has been applied in mouse (de Solis *et al.*, 2016) and human IPSCs (González *et al.*, 2014). Alternatively, the Cas9/dCas9 protein can be allosterically regulated via the insertion of the ligand binding domain of estrogen receptor. The Cas9/dCas9 is therefore dependent on the ligand specific binding of tamoxifen. The process is reversible as Cas9/dCas9 activity could be switched off upon removal of the ligand (Oakes *et al.*, 2016). Neither of these systems have been set up in zebrafish so far.

In summary, these new transgenic lines were found to be able to drive different Hdac1 constructs in the spinal cord ERGs. The Tet-On system provides spatial and temporal control to the expression of the constructs in both the larval and adult zebrafish spinal cord. Additional characterisation of the *Her4.1*:TetA:TetRE:dnHdac1 line is required

to improve the expression in adulthood. The ability of both lines to affect acetylation levels has not been determined yet. However, the two new transgenic lines were carried forward into neuroregeneration assays to see if biological effects could be observed.

Chapter 4 Acetylation and Hdac1 in the unlesioned spinal cord

4.1 Introduction

Neurogenesis is mainly complete by 3 dpf in the zebrafish spinal cord. A colabelling study with motor neuron markers and proliferative marker EdU was used to birth date when motor neurons were generated in the spinal cord (Reimer *et al.*, 2013). EdU was exposed to embryos at different time points and analysed for co labelling with *Islet1*:GFP^{+} or Hb9^{+} motor neurons at 72hpf. As shown in Figure 4.1 almost all motor neurons are born by 51hpf. At 3dpf the pMN ERGs in the spinal cord has mostly stopped generating motor neurons, though they still generate oligodendrocytes (Park *et al.*, 2005).

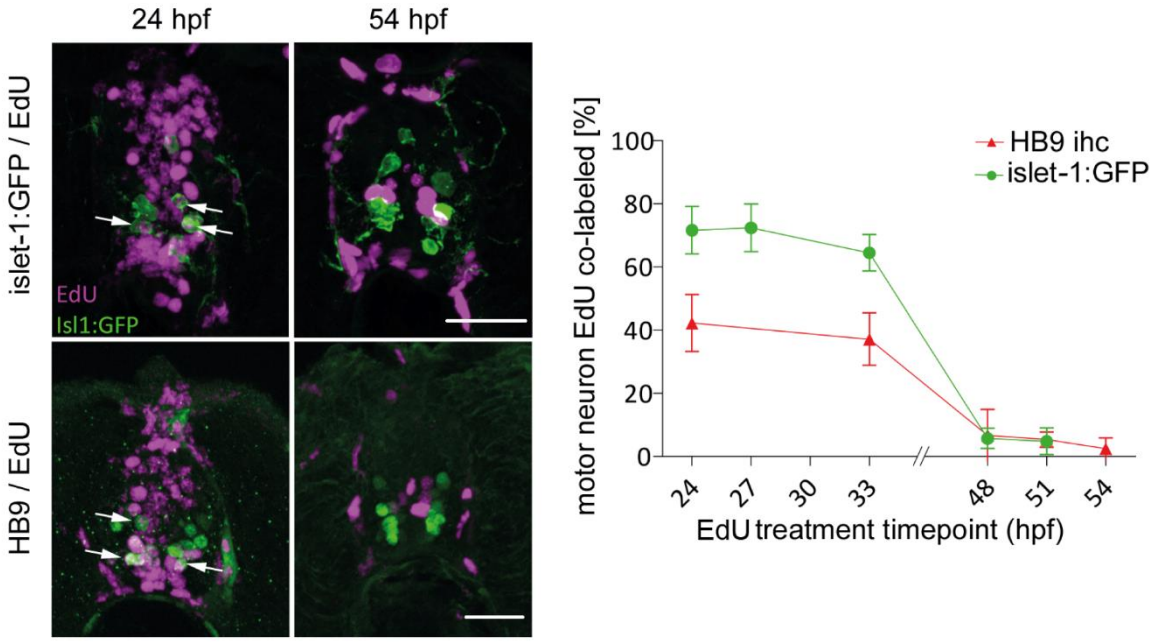


Figure 4-1: Neurogenesis is complete by 3pf. Spinal cord cross sections of embryos at 72hpf after exposure to EdU (magenta) at different timepoints;24hpf or 54hpf. Arrows indicate double labelled cells. Scale bars are 60µm. Graph shows the number of double labelled cells at 72hpf after EdU exposure at different timepoints (x-axis). At least 6 larvae analysed per timepoint. Adapted from supplemental figure 1 in (Reimer *et al.*, 2013).

Further work into the unlesioned larval spinal cord revealed that between 3 to 5dpf ~2.5 motor neurons (*Hb9*:GFP/EdU) were born. Using EdU acutely to label proliferation found that at 4dpf ~3 pMN cells had proliferated in the unlesioned spinal cord. Both these measurements increased after a lesion significantly (Ohnmacht *et al.*, 2016). Therefore, the larval spinal cord at 3dpf can be used to model the intact quiescent spinal cord.

In Chapter 1 acetylation was shown to be able to regulate developmental neurogenesis. Components of the acetylation machinery have also been found to be able to modulate the progenitor behaviour during adult neurogenesis. A HAT from the MYST family, Querkopf was found to be necessary in mammalian ERGs in adulthood. Querkopf- deficient ERGs had reduced self renewal capacity and reduced ability to produce neurons (Merson *et al.*, 2006). Removal of HDAC2 did not affect developmental neurogenesis but reduced neurogenesis in adulthood (Jawerka *et al.*, 2010). Conditional knockout of HDAC3 in adulthood reduced hippocampal progenitor proliferation (Jiang and Hsieh, 2014). The effect of acetylation on adult neurogenesis in the zebrafish spinal cord has not been investigated so far.

As discussed in Chapter 1, many of the extrinsic signals that can modulate the behaviour of the spinal cord ERGs after injury do not have effects in the unlesioned spinal cord. This suggests some still unknown injury induced change that occurs in the ERGs which changes their receptiveness to the subsequent extrinsic signals. To assess whether increase in acetylation possibly modulated by Hdac1 changes could be a such unknown signal, acetylation was manipulated using pharmacological agents and the newly generated transgenic lines to see if motor neuron generation could be stimulated after the normal time frame of development.

4.2 Results

4.2.1 HAT inhibition in the unlesioned spinal cord promotes neurogenesis

Deacetylation by HDAC enzymes is countered by the activity of the HAT enzymes. The HATs add on acetyl groups to lysine residues. HAT inhibition and an increase in Hdac1 activity will lead to the same outcome – hypoacetylation. Therefore, HAT inhibition can be used as an indirect method to mimic Hdac1 induced deacetylation. EML425 inhibits the CBP/p300 HAT enzyme (Milite *et al.*, 2015). This particular HAT was chosen as it counteracts Hdac1 deacetylation of targets such as Gli transcription factors (Coni *et al.*, 2013). To test whether a decrease in acetylation in the unlesioned spinal cord could be a signal in the progenitor to start the neurogenic process, *Hb9*:GFP larvae were treated with EML425 at 3dpf. At this time point developmental motor neuron generation is strongly diminished (Reimer *et al.*, 2013). The numbers of double positive *Hb9*:GFP/EdU cells, in three somite segments at the end of the yolk extension, was assessed at 5dpf. EML425 at 2µM did not significantly change the numbers though there was a trending increase (Vehicle 4.864±1.068; EML425 7.476±1.075, data not shown), therefore the concentration was increased to 3µM. Larvae treated with EML425 at 3µM showed a 93.2% increase in the number of new

born motor neurons (Vehicle 5.13±0.6722; EML425 9.913±0.8456, Figure 4.2D). Due to the unusual presence of some dorsal double labelled cells, the location of the cells was assessed, and the numbers of double labelled cells were divided on their location in the spinal cord. Cells that were located high in the spinal cord above the normal ventral band of cells were termed as dorsal (indicated with white arrow heads in Figure 4.2C). The numbers of double positive *Hb9*:GFP/EdU cells in both the ventral and dorsal locations in the spinal cord were significantly increased after EML treatment (Ventral: Vehicle 4.714±0.6292; EML 6.478±0.485 and Dorsal: Vehicle 2.952±0.4755; EML 6.565±0.6968, Figure 4.2E).

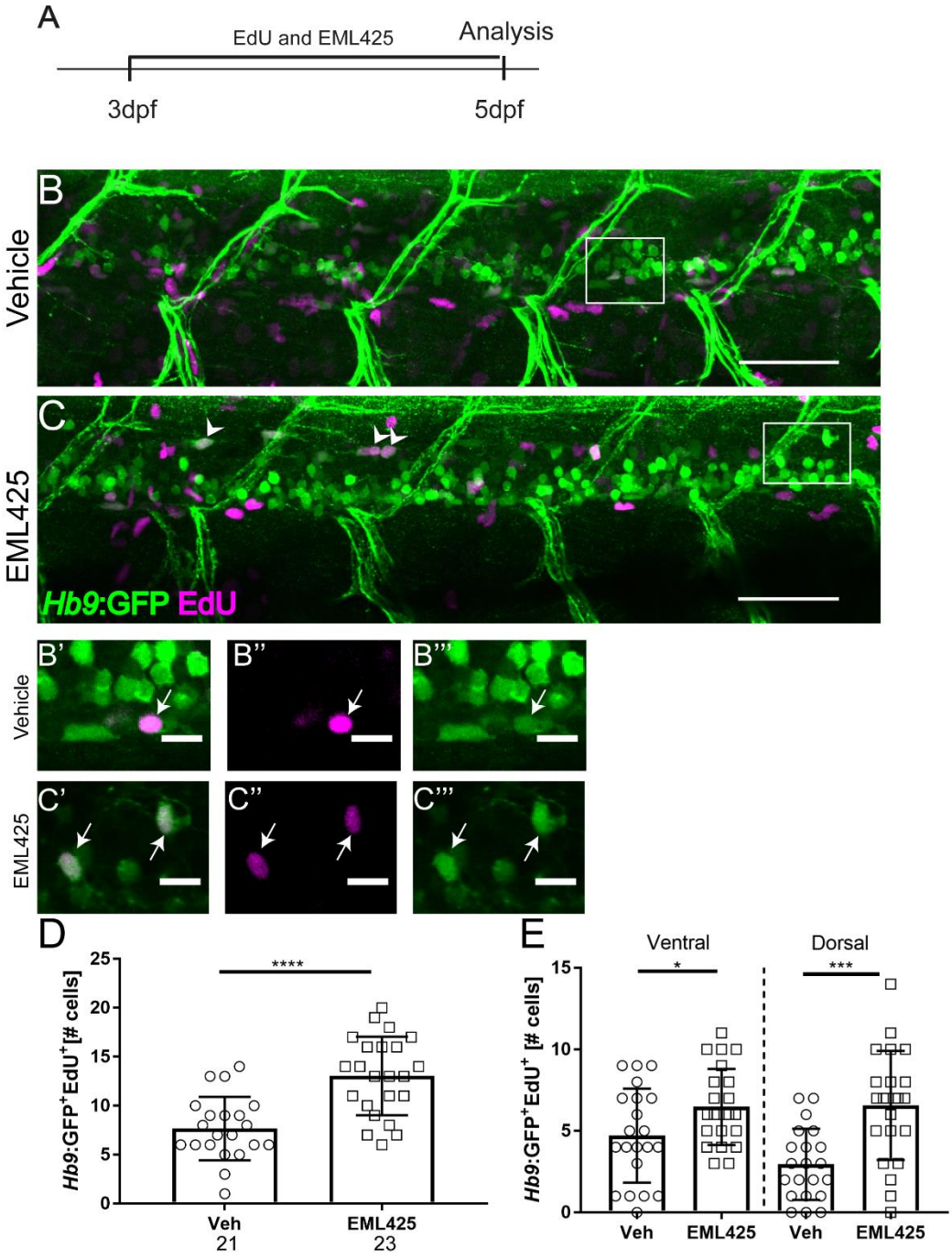


Figure 4-2: HAT inhibition in the unlesioned spinal cord increases the number of new born motor neurons. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Hb9*:GFP (green) larvae at 2 days post treatment labelled with EdU (magenta). Lateral views of the trunk region are shown. White arrow heads in C point to dorsal double positive cells. Scale bars are 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D-E**; Quantification of the number of double positive *Hb9*: GFP and EdU cells in three somite segments. Data shown as mean±SEM as bar and scatter plot. N numbers are written below, each dot is a fish; 2 independent experiments. D is total number and E is the data split on

location of the cells in the ventral-dorsal axis. Unpaired T-test, ****P<0.0001, *P=0.0305, ***P=0.0001, Post hoc power analysis: 0.99.

4.2.2 Hdac1 overexpression in progenitors in the unlesioned spinal cord had no effect on neurogenesis

HAT inhibition is not a direct method to test the effect of an increase in Hdac1 expression as the HAT pharmacological inhibitor is not a cell specific manipulation and may be acting on other cells apart from the spinal cord ERGs. Secondly, the cellular targets of the manipulations may be different. Since there is no pharmacological agent that can increase Hdac1 activity directly a genetic approach to overexpress Hdac1 was used. The new transgenic TetResponder was used to investigate whether increasing Hdac1 levels in the progenitors would have the same effect. *Her4.1*:TetA;TetRE:YFP-Hdac1 double transgenic animals crossed to *Mnx1*:RFP reporter fish were induced and treated with EdU at 3dpf. The numbers of double positive *Mnx1*:RFP/EdU cells was assessed at 5dpf similar to the analysis performed on the Hb9:GFP line after EML425 treatment. Unlike in the *Hb9*:GFP transgenic line, the control *Mnx1*:RFP larvae had no double labelled cells, indicating no neurons labelled by this transgene were born during this timeframe . This did not change when progenitors expressed *hdac1* for 2 days (Doxycycline 0; *hdac1* 0, Figure 4.3D).

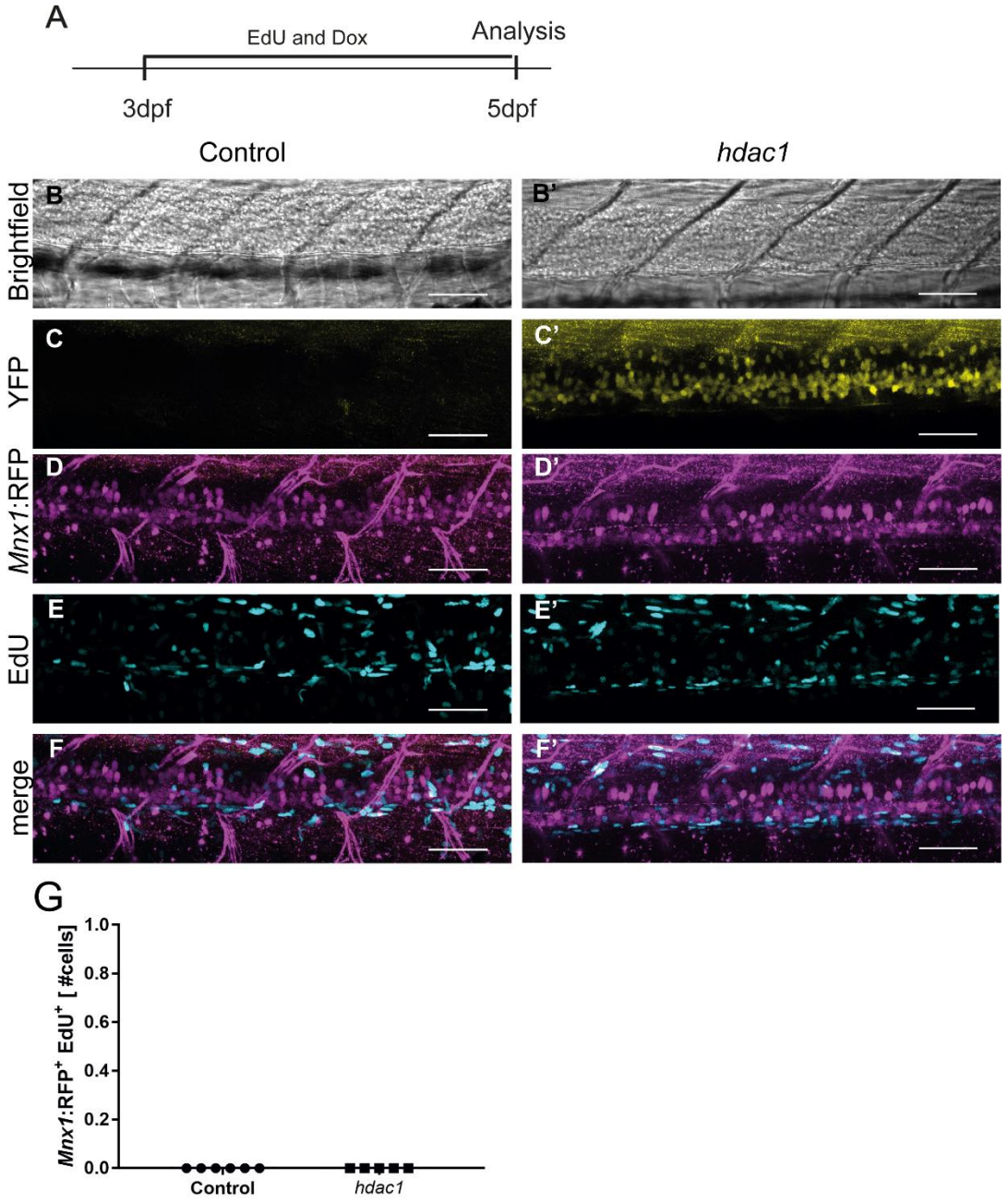


Figure 4-3: Overexpression of Hdac1 in progenitors in the unlesioned spinal cord did not increase numbers of new born motor neurons. A; Timeline of the experiment. **B-F'**; Representative maximal intensity projections of *Mnx1*:RFP (magenta) ;*Her4.1*:TetA;TetRE:YFP-Hdac1(yellow) at 2 days post induction labelled with EdU (cyan). The different channels are shown separately (B-E') and merge of EdU and *Mnx1*:RFP (F,F'). Lateral views of trunk region are shown. Scale bars are 50µm. **G**; Quantification of the number of double positive *Mnx1*:RFP and EdU cells in three somite segments. Data shown as mean±SEM. N=6/5 animals; 1 independent experiment.

4.2.3 Hdac1 overexpression in the progenitors in the unlesioned spinal cord increases progenitor proliferation

Though neurogenesis was not observed to be altered the activity of the progenitors may still be changed by *hdac1* overexpression. I used phospho-histone 3 (pH3) immunohistochemistry to label the cells that are undergoing mitosis. *Her4.1*:TetA; TetRE:YFP-Hdac1 animals were induced at 3dpf. The AmCyan label of the Tet activator line was used to label the spinal cord ERGs. The numbers of AmCyan positive cells that were pH3 positive at 1 and 2 days post induction was assessed. The controls were age matched uninduced animals; at 4 and 5dpf respectively. At 1 day post induction no significant difference was found between the groups (Control 0.3±0.1051, *hdac1* 0.2±0.09177, Figure 4.4G). At 2 days post induction the larvae expressing *hdac1* had a 237.3% increase in the number of progenitors in mitosis (Control 0.2353±0.106, *hdac1* 0.7931±0.1674, Figure 4.4G).

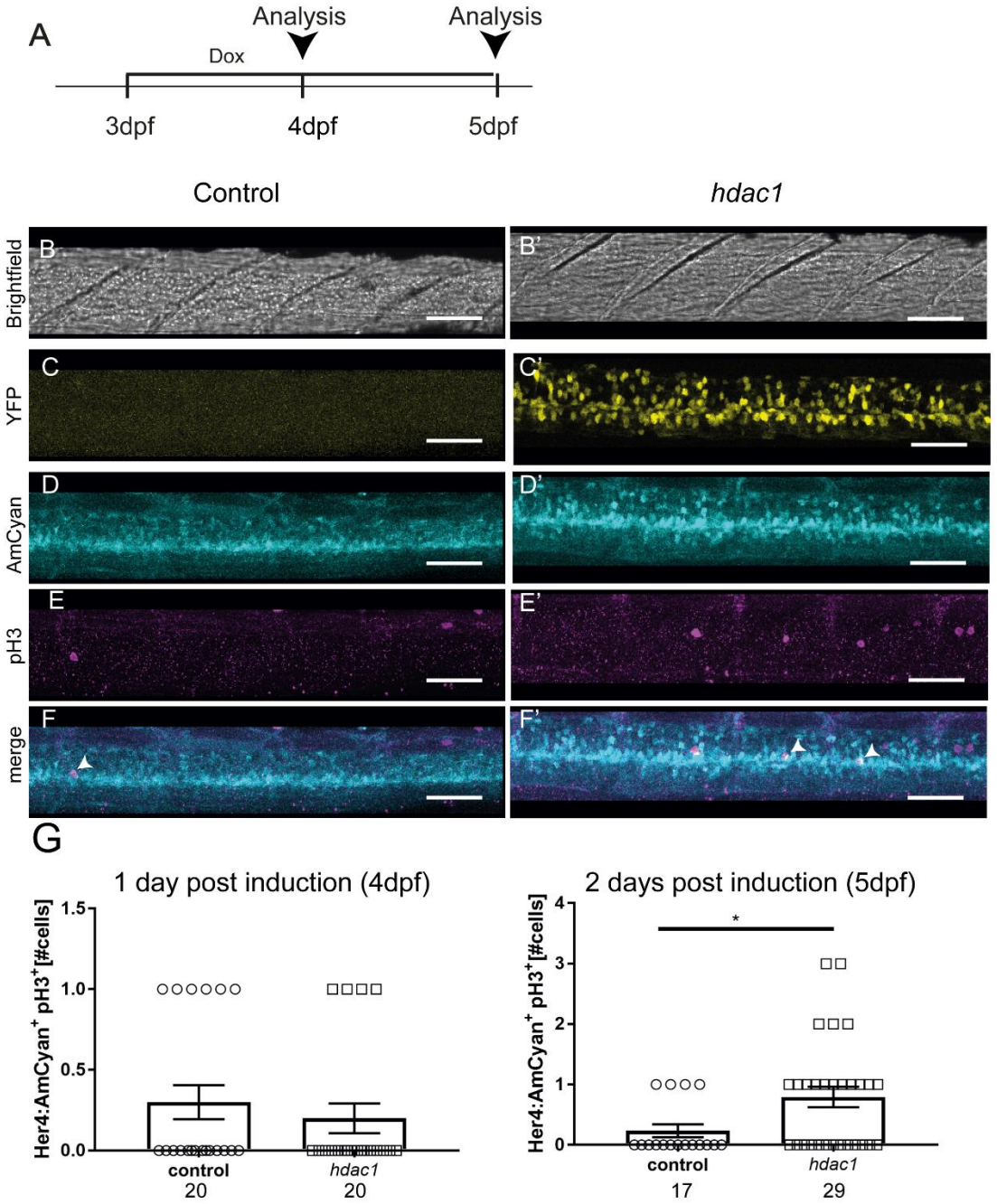


Figure 4-4: Expression of *hdac1* in the unlesioned spinal cord increased progenitor proliferation. A; Timeline of experiment. **B-F'**; Representative maximal intensity projections of *Her4.1*:TetA;TetRE:YFP-Hdac1 (cyan) larvae at 2 days post induction labelled with anti-pH3 (magenta). The different channels are shown separately (B-E') and merge of pH3 and AmCyan (F,F'). White arrow heads indicate double positive cells. Lateral views are shown. Scale bars are 50µm.**G**; Quantification of the number of double positive AmCyan and pH3 cells in three somite segments at 1 and 2 days post induction. Data shown as mean±SEM as bar and scatter plot. N numbers are written below; 3 independent experiments. Mann-Whitney, *P=0.0252, Post hoc power analysis: 0.71.

4.3 Discussion

In this Chapter deacetylation was investigated as a mechanism for stimulating ERGs to begin neurogenesis after the standard development window has past. To do this I used the newly generated transgenic line that conditionally expressed Hdac1 specifically in the spinal cord ERGs and the pharmacological inhibition of the HAT enzymes. The results obtained are summarised in Table 4.1.

Table 4-1: Summary of results in chapter 4. Results for different manipulations are separated by cellular readout. ↑significant increase, ─ no change and blank space experiment was not performed in this study.

	Hdac1	EML425
Motor Neurons	─	↑
Total ERG proliferation	↑	

4.3.1 Deacetylation in the absence of a lesion is sufficient to induce motor neuron generation

HAT inhibition in the unlesioned larval spinal cord increased neurogenesis. During zebrafish development more than 95% of the motor neurons are born by 54hpf and after 3dpf only 0.5% of the motor neuron population are produced (Reimer *et al.*, 2013). EML425 treatment at 3µM between 3 and 5dpf increased the numbers of new born motor neurons. This result suggested that deacetylation in the ERGs may promote them to begin producing neurons again. However, the expression of *hdac1* in the ERGs did not show the same increase in neurogenesis. The inconsistent results between *hdac1* overexpression and HAT inhibition could be due to the differential labelling of the *Mnx1*:RFP transgenic line compared to the *Hb9*:GFP line. The GFP transgenic lines cannot be combined with the Tet-On lines as the YFP expression cannot be separated from the GFP expression. Labelling all neurons using HuC immunohistochemistry, as performed in the lesioned conditions (detailed in chapter 5), would allow further examination of the effect of *hdac1* overexpression on neurogenesis in the unlesioned spinal cord.

Hdac1 cannot be overexpressed in the ERGs in the unlesioned adult spinal cord in the model that was generated in this study (as detailed in chapter 3). However, EML425 treatment could be used to investigate whether deacetylation causes the ERGs in the adult unlesioned spinal cord to generate neurons as it did in the larval spinal cord. This could help separate from the possibility that the larval ERGs are still in a development mode. The larval spinal cord at 3dpf has stopped generating motor

neurons but other cell such as interneurons are still being generated at this time point (Briona and Dorsky, 2014; Ohnmacht *et al.*, 2016). The pMN domain is not completely quiescent at this stage either as it is generating mostly oligodendrocytes (Park *et al.*, 2005; Czopka, ffrench-Constant and Lyons, 2013). A spinal lesion at 3dpf declines this oligodendrogenesis in favour of motor neurogenesis (Ohnmacht *et al.*, 2016). It would be interesting to observe if *hdac1* overexpression/deactylation also decreases oligodendrogenesis while increasing neurogenesis.

4.3.2 Hdac1 expression in unlesioned spinal cord increased progenitor proliferation

Though Hdac1 expression in the ERGs did not change neurogenesis as measured in this study there was an increase in the number of ERGs in mitosis. The pH3 label was used in combination with *Her4.1*:AmCyan to see the rate of proliferation of the entire ERG population at 4 or 5dpf. This is a different labelling strategy to what was used previously (Ohnmacht *et al.*, 2016) but demonstrated that the unlesioned larval spinal cord is indeed in a highly quiescent state as there was very little double labelling in the control conditions. This measurement also increased after a lesion (shown in chapter 5). 2 days of *hdac1* expression in the absence of a lesion was able to significantly increase this low rate of proliferation. Further experiments looking into additional methods of detecting proliferation would be of interest e.g. acute EdU treatment and PCNA immunohistochemistry, to confirm the pH3 result shown here. Different reporter lines labelling the spinal cord ERGs should be used as the AmCyan could not be enhanced by antibodies and the signal was not always of sufficient strength for analysis. These results suggest the possible role that deacetylation has in stimulating the ERGs out of their quiescent state. This would be the first manipulation to stimulate spinal cord ERGs in the unlesioned context.

In summary, deacetylation possibly achieved through a change in Hdac1 expression may be a trigger for spinal cord ERGs to leave their quiescent state and contribute to neurogenesis. To further investigate this the new transgenic lines were used to test whether Hdac1 was a positive modulator of neurogenesis in the spinal cord after injury.

Chapter 5 Acetylation and HDAC1 in the lesioned spinal cord

5.1 Introduction

Changes in acetylation have been observed in many different tissues after injury (Lv *et al.*, 2011; Finelli, Wong and Zou, 2013; Huang, Barr and Rudnick, 2013) . These changes could be due to changes in HDAC activity or expression in cells after the injury (Zhang *et al.*, 2012; Huang, Barr and Rudnick, 2013; Jablonska *et al.*, 2016). Whether Hdac1 is a positive or negative regulator of regeneration is unclear with the results often depending on the cell type involved and the techniques used. Here I discuss the studies that have investigated the role of Hdac1 in regeneration in tissues outside and inside the nervous system.

5.1.1 HDAC in repair outside the nervous system

After acute kidney injury in mice, Class I HDAC inhibition impaired renal regeneration (Tang *et al.*, 2014) . In liver injury models in mice the expression of several HDACs was increased and treatment with HDAC inhibitors delayed regeneration (Ke *et al.*, 2012; Huang, Barr and Rudnick, 2013). The appropriate expression levels of HDAC was shown to be necessary for repair, however, as when HDAC1 was overexpressed the livers show increased proliferation and failed to stop the regeneration process (Jin *et al.*, 2015). HDAC1/2 double knockout in secretory cells using Scgb1a1-Cre, in the mouse lung epithelium showed reduced regeneration of the secretory epithelial cells and reduced levels of proliferation after naphthalene injury. The effect was long lasting as the lack of regeneration was also observed after 1 month. They suggest that HDAC1/2 regulates the process through repression of the cell cycle inhibitor Rb1. The effect of HDAC1/2 knockout was only seen after an injury meaning HDAC1/2 had a specific role in the regeneration process of these cells (Wang *et al.*, 2013). In contrast, other organ systems have evidence for the negative role of HDAC in regeneration. In the heart, systemic treatment with pan-HDAC inhibitor TSA after myocardial infarction led to an increase in proliferation, better functional outputs and overall increased survival of the mice (Zhang *et al.*, 2012).

In the non-mammalian models, such as *Xenopus* and zebrafish, Hdac1 mRNA expression is detected in the regenerating tail and caudal fin after amputation. Treatment with pharmacological inhibitors lead to reduced proliferation and regenerative outgrowth (Tseng *et al.*, 2011; Taylor and Beck, 2012; Pfefferli *et al.*, 2014). Hdac inhibition also impairs regeneration of the axolotl tail and *Xenopus* limb

(Taylor and Beck, 2012). In the zebrafish caudal fin, Hdac1 function was necessary for osteoblast redifferentiation during regeneration and HDAC inhibition changed the expression of genes related to pluripotency and dedifferentiation during regeneration (Pfefferli *et al.*, 2014).

5.1.2 HDAC in repair in the Peripheral nervous system

In the peripheral nervous system HDAC1/2 was found to be upregulated in the Schwann cells of adult mice after nerve crush injury. Genetic ablation of HDAC1/2 in the Schwann cell, using P0-CreERT2, found that remyelination after injury was decreased but axon growth was promoted. This removal of HDAC1/2 caused changes in gene expression leading to a more pro-repair phenotype of the Schwann cells. Mice treated with Class I-specific inhibitor Mocetinostat showed improved performance on the rotarod and toe pinch test after injury. This effect was time dependent, as longer treatment did not show the same improvement (Brügger *et al.*, 2017). Sciatic nerve lesion in mice increases acetylation levels in the dorsal root ganglion neurons. ChIP assays with antibodies against AcH4 found it is enriched at the promoters of regeneration associated genes (RAGs) induced by a conditioning lesion. Treatment with HDAC inhibitors was sufficient to increase expression of a subset of these RAGs (Finelli, Wong and Zou, 2013). However, the expression of nearly half (48%) of the RAGs did not change after HDAC inhibition and the fold changes were not to the same extent as induced by conditioning lesion. In contrast another study found that the HDAC-NuRD complex is recruited by the Zeb2 transcription factor in Schwann cells which is necessary for remyelination after injury (Wu *et al.*, 2016).

In the zebrafish lateral line, TSA and VPA treatment inhibited sensory hair cell regeneration. This decrease in regeneration was through decreasing progenitor cell proliferation and not changing the rate of cell death (He *et al.*, 2014). In the avian inner ear, the sensory hair cells are capable of regeneration. Treatment with HDAC inhibitors of cultured utricles, after aminoglycoside antibiotic ablation of the hair cells, led to a reduction in proliferation and a reduction in number of new hair cells when inhibited for the entire recovery time (Slattery, Speck and Warchol, 2009).

5.1.3 HDAC in repair in the Central nervous system

In the central nervous system many studies have examined the role of HDAC in regeneration after injury. The activity of all HDACs was increased in white matter tissue after hypoxia in mice. The sirtuins (class III HDACs) were found to be necessary

for oligodendrocyte precursor cell proliferation and differentiation (Jablonska *et al.*, 2016). After stroke HDAC activity has a negative role in repair as HDAC inhibition reduced infarct volume and improved performance on eight-point behavioural test and the rotarod (Kim *et al.*, 2007). In the adult rodent retina the overexpression of neurogenic transcription factor Ascl1 in Müller glia and subsequent treatment with TSA led to an increase in neural markers in the retinas after NMDA damage (Jorstad *et al.*, 2017). TSA also increased retinal axonal outgrowth through the induction of RARβ expression (Koriyama *et al.*, 2014). After rodent spinal cord injury a decrease in acetylation levels was observed from 1 day post lesion and continued for the 2 weeks assessed (Lv *et al.*, 2011). Treatment with HDAC inhibitors after spinal cord injury led to reduced levels of apoptosis, better locomotion function (Lv *et al.*, 2011; Chu *et al.*, 2015; Zhang *et al.*, 2018), increased levels of progenitor markers nestin and sox2 (Bang *et al.*, 2013) and neuronal markers DCX and NeuN (Chu *et al.*, 2015; Zhang *et al.*, 2018). When neurospheres were made from progenitor cells from the injured spinal cords VPA treatment was found to decrease the cells proliferation ability and increase their neurodifferentiation (Chu *et al.*, 2015). In sensory spinal cord injury model, treatment with HDAC inhibitors, TSA and MS-275, increased the number of regenerating axons, labelled with DexTR tracer, in the lesion site without effecting glia scar volume, defined by dense $GFAP^+$ immunohistochemistry (Finelli, Wong and Zou, 2013).

In lamprey, after spinal cord injury, neurons with higher Hdac1 expression showed higher regenerative abilities than neurons with lower Hdac1 levels (Chen, Laramore and Shifman, 2016). In adult zebrafish, Hdac1 was found to be differentially expressed at different time points after spinal cord injury using genome wide expression profiling (Hui *et al.*, 2014). They observed two peaks of increased expression at 3 and 15 days post lesion but do not examine in which cell types this increase occurs. After spinal cord injury in both adult and larval zebrafish, previous work by our group has shown that Hdac1 mRNA levels are increased at the injury site in the spinal cord progenitor cells. After a lesion in the adult spinal cord the peak of motor neuron production and progenitor proliferation is at 14 days post lesion (dpl) (Reimer *et al.*, 2008). *Hdac1* expression was assessed by in situ hybridisation on spinal cord sections at this 2 week time point. *Hdac1* is upregulated in the ventricular zone, where the ERGs reside, compared to the expression observed in the unlesioned spinal cord (Figure 5.1 A). After a lesion in the larval spinal cord, regeneration of motor neurons occurs within two days (Ohnmacht *et al.*, 2016). *Hdac1* was assessed at both 24 and 48 hours post

lesion. Fluorescent-activated cell sorting was used to separate out progenitor cells using the *Her4.1*:GFP transgenic zebrafish line, which labels the ERGs with GFP. GFP positive cells were used for RNA extraction and levels of *Hdac1* were measured using quantitative PCR. *Hdac1* expression significantly increases by 50% at 24 hours post lesion and returns to unlesioned levels by 48 hours post lesion (Figure 5.1 B).

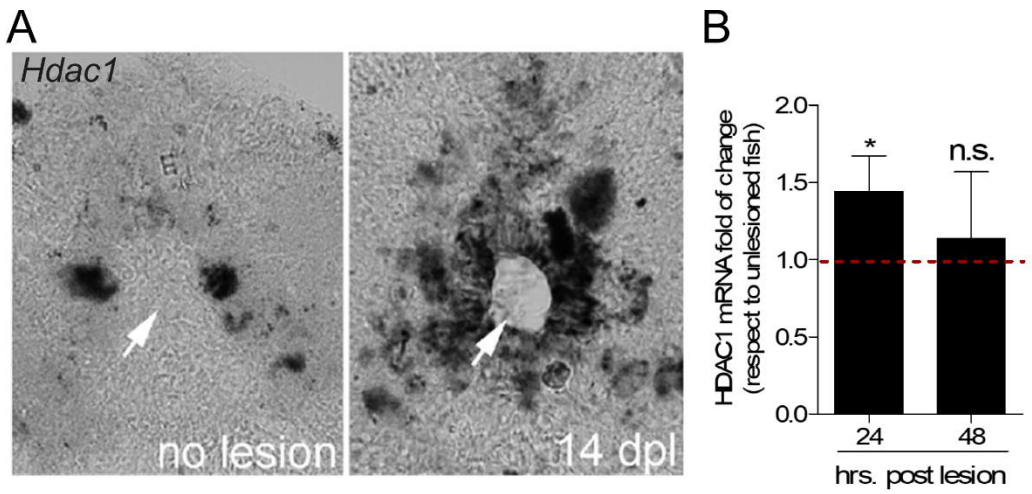


Figure 5-1: Hdac1 expression is increased in zebrafish spinal cord after lesion. A; Hdac1 mRNA expression in adult spinal cord sections unlesioned (left) and 14 days post lesion (right) by in situ hybridisation. **B**; Hdac1 expression from Her4.3:GFP sorted cells larval zebrafish at 24 and 48 hours post lesion. Generated by Dr. Karolina Mysiak and Dr. Leonardo Cavone.

Since Hdac1 expression increased during the time window of regeneration, whether Hdac function was necessary for regeneration in larval zebrafish was tested. Larvae zebrafish were lesioned and then were treated with either the pan-HDAC inhibitor TSA (200nM) or Class 1- specific inhibitor Mocetinostat (1µM). The concentrations were chosen as were non toxic to the larvae and were found to change acetylation level. In both treatment conditions there is a significant reduction in the number of new born motor neurons (Vehicle 3.271±0.2475; TSA 0.8163±0.1478, Figure 5.2A) (Vehicle 2.421±0.2993; Mocetinostat 1±0.2182, Figure 5.2B).

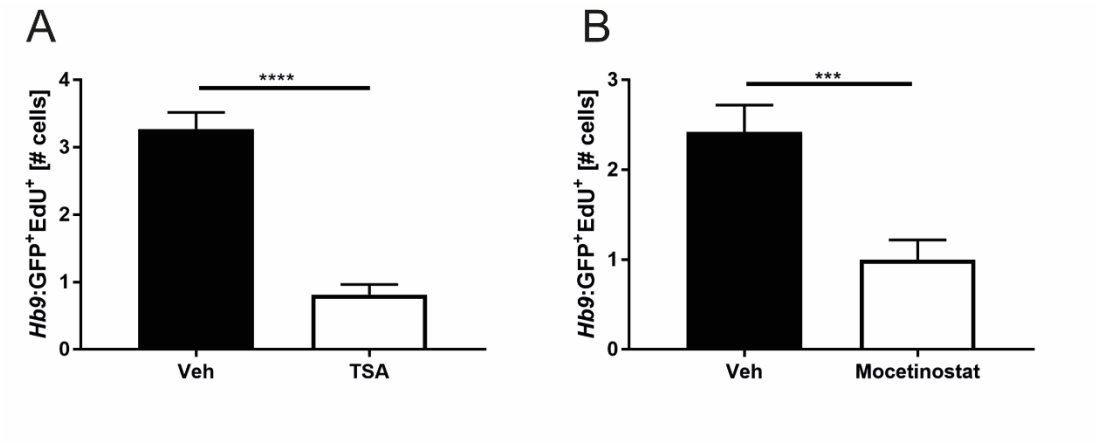


Figure 5-2: Motor neuroregeneration is inhibited by HDAC pharmacological inhibition. **A-B**; Quantification of the number of double positive *Hb9*:GFP and EdU cells at 2 days post lesion after treatment with 200nM TSA (A) or 1µM Mocetinostat (B). Data shown as mean±SEM. Mann-Whitney test, ****P<0.0001,***P=0.0002. Generated by Dr. Leonardo Cavone.

Therefore, the evidence collected so far suggests that Hdac has a positive role in neuroregeneration in the zebrafish spinal cord.

5.1.4 Limitations of previous research

Systemic treatment with HDAC inhibitors in models of spinal cord injury in mammals have been found to improve regeneration (Lv *et al.*, 2011; Bang *et al.*, 2013; Chu *et al.*, 2015; Zhang *et al.*, 2018) whereas our group has found evidence that HDAC inhibitors inhibit neuroregeneration in the zebrafish spinal cord. There are several possibilities for the discrepancies. Firstly, many of these studies in mammals do not address the effects these treatments may have in the numerous cell types present at the lesion site after an injury. Cells of the immune system have been found to express HDACs after spinal cord injury, for example HDAC3 (Kuboyama *et al.*, 2017). Treatment with RGFP966, a highly specific HDAC3 inhibitor, in mice was found to have beneficial effects in spinal cord injury through modulating the immune response. Class I- specific inhibitor CI 1994 treatment after spinal cord injury was found to supress neutrophil recruitment and decrease cytokine levels (Zhang *et al.*, 2018). The HDAC inhibitors could, therefore, not be acting directly on ERGs but through indirect means by altering the immune response to the injury.

Secondly, some HDAC inhibitors have many intracellular targets. The authors do not rule out the effect these compounds may have on these additional cellular targets. Valproic acid (VPA) can inhibit HDACs and GABA transaminase (Baldino and Geller,

1981). VPA treatment would lead to increased GABA levels. GABA receptors are expressed by progenitors in the rodent brain (Nguyen *et al.*, 2003; Liu *et al.*, 2005; Tozuka *et al.*, 2005). Modulation of GABA signalling alters proliferation and neurodifferentiation in the rodent brain (Nguyen *et al.*, 2003; Liu *et al.*, 2005; Fernando *et al.*, 2011). Inhibition of GABA facilitates muller glia proliferation in the zebrafish retina (Rao, Didiano and Patton, 2017). Increasing GABA signalling in lamprey after spinal cord injury promotes axonal regeneration (Romaus-Sanjurjo *et al.*, 2018) but the effect of this neurotransmitter on neuroregeneration in the mammalian and zebrafish spinal cord is unknown.

In summary, HDACs are present in all cell types and can have varied effects depending on the cell type in question. When looking at regeneration *in vivo*, a cell specific manner of manipulating HDAC levels is needed to avoid any compounding issues using the pharmacological inhibitors and to fully understand what HDAC is doing in the cell process.

5.2 Results

5.2.1 Doxycycline treatment does not affect the immune response after injury

Since this system was to be used to avoid the potential effects that HDAC inhibitors have on the immune system, the effect of doxycycline on the immune response to spinal cord injury was tested. *Mpeg1*:GFP larvae were lesioned and then treated with the same doxycycline concentration that is used to induce gene expression (485nM). This reporter which labels the macrophages and microglia with green fluorescent protein (Ellett *et al.*, 2011). The numbers of GFP positive cells were recorded at 24 hours post injury. This timepoint was chosen as it is when the highest number of macrophages are at the lesion site (Tsarouchas *et al.*, 2018). No significant difference in number of *mpeg1*:GFP cells was found between treatment groups (Vehicle 28.9± 2.008 ; Doxycycline 34.6±2.099, Figure 5.3). The number of macrophages for both groups are in line with previous observations (Tsarouchas *et al.*, 2018).

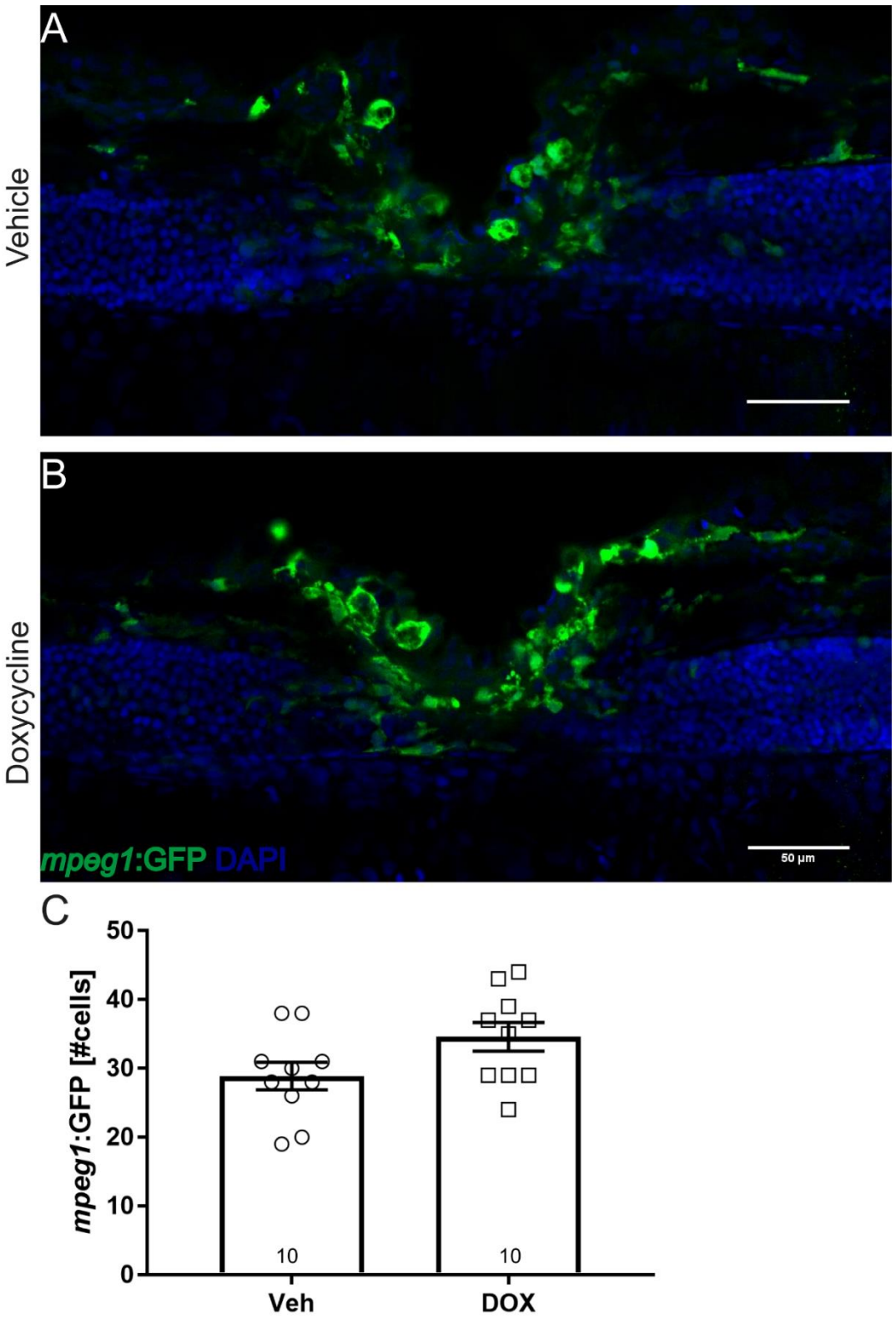


Figure 5-3: Doxycycline treatment does not affect the number of macrophages at the lesion site. **A-B**; Representative pictures of *mpeg1*:GFP (green) at the lesion site, with DAPI (blue) to show the spinal cord. **C**; Quantification of number of *mpeg1*:GFP cells within 200µm of lesion site at 24hpl. Data shown as mean±SEM, as a bar and scatter plot. N numbers shown

on graph, each dot is a fish; 1 independent experiment. Unpaired T-test, P=0.0654, A priori power analysis: 22 animals/group.

5.2.2 Doxycycline treatment alone does not affect neuroregeneration

To control for possible toxic effects of doxycycline treatment on regenerative neurogenesis, I lesioned single transgenic *Mnx1*:RFP larvae and treated with doxycycline (485nM). This reporter line labels the motor neurons with red fluorescent protein (Jao, Appel and Wente, 2012). The thymidine analogue EdU was used to label the new born cells. EdU is an alternative for the BrdU assay and is incorporated into DNA during active DNA synthesis (Salic and Mitchison, 2008). The number of new born motor neurons was then assessed at 2 days post lesion by counting the number of double positive *Mnx1*:RFP/EdU cells around the lesion site. This timepoint was chosen as this when a significant increase in new born motor neurons had been previously observed (Ohnmacht *et al.*, 2016). There is a lesion induced increase in number of doubled labelled cells when compared to levels measured in the unlesioned spinal cord shown in Chapter 4 (Unlesion 0; Vehicle 1.857±0.3099;*P=0.0286). No significant difference in the number of double labelled cells was found after doxycycline treatment (Vehicle 1.857±0.3099; Doxycycline 2.121±0.3935, Figure 5.4). This indicates that doxycycline treatment alone does not negatively impact the immune system response to injury or regenerative neurogenesis. Doxycycline, therefore, can be used to induce gene expression in larval zebrafish using the Tet-On system.

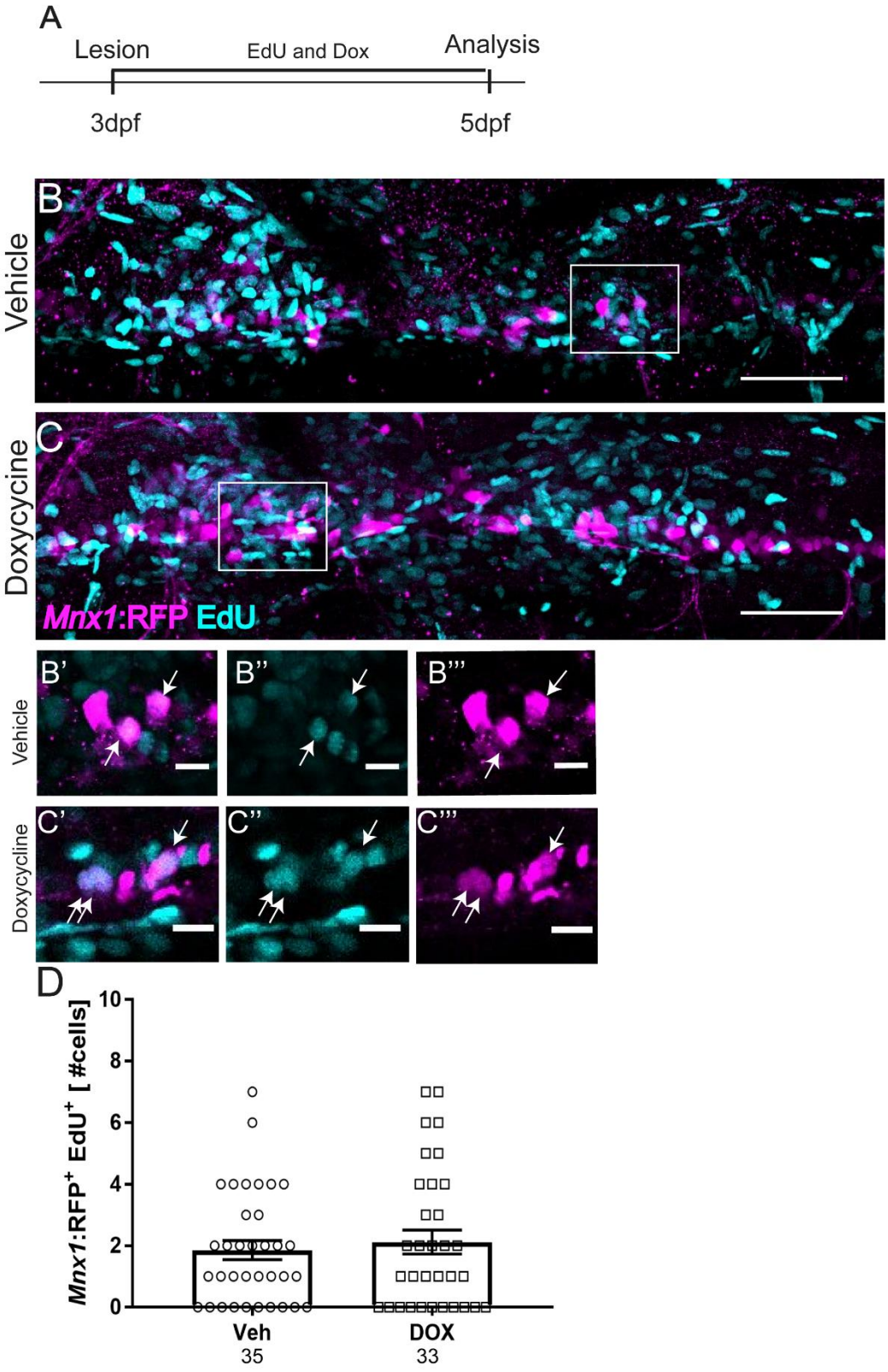


Figure 5-4: Doxycycline does not affect neuroregeneration. A; Timeline of experiment. **B-C**; Representative maximal intensity projections of *Mnx1*:RFP (magenta) larvae at 2dpl labelled with EdU (cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars

are 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the double positive *Mnx1*:RFP and EdU cells in 250µm around the lesion site. Data is shown as man±SEM, as bar and scatter plot. Total N numbers are written below, each dot is a fish; 3 independent experiments. Mann-Whitney test, P=0.8733, A priori power analysis: 955 animals/group

5.2.2 Cell specific expression of dnHdac1 reduces motor neuron regeneration after a lesion in larval zebrafish

Since the Hdac pharmacological inhibition after a lesion had reduced regenerative neurogenesis we next tested test whether inhibition of Hdac1 activity in the progenitor cell was sufficient to also reduce numbers of new born neurons after a lesion. Double transgenic *Her4.1*:TetA;TetRE:YFP-dnHdac1 fish crossed to *Mnx1*:RFP reporter fish were lesioned at 3dpf and induced with doxycycline. EdU was placed into the water and the number of double labelled *Mnx1*:RFP/EdU cells was assessed at 2 days post lesion. Controls were fish that were from the same clutch that were treated with doxycycline but had no YFP expression. Larvae that were expressing *dnhdac1* had a 46% reduction in the number of new born motor neurons after a lesion (Control 2.545±0.3977; dnHdac1 1.353±0.3191, Figure 5.5).

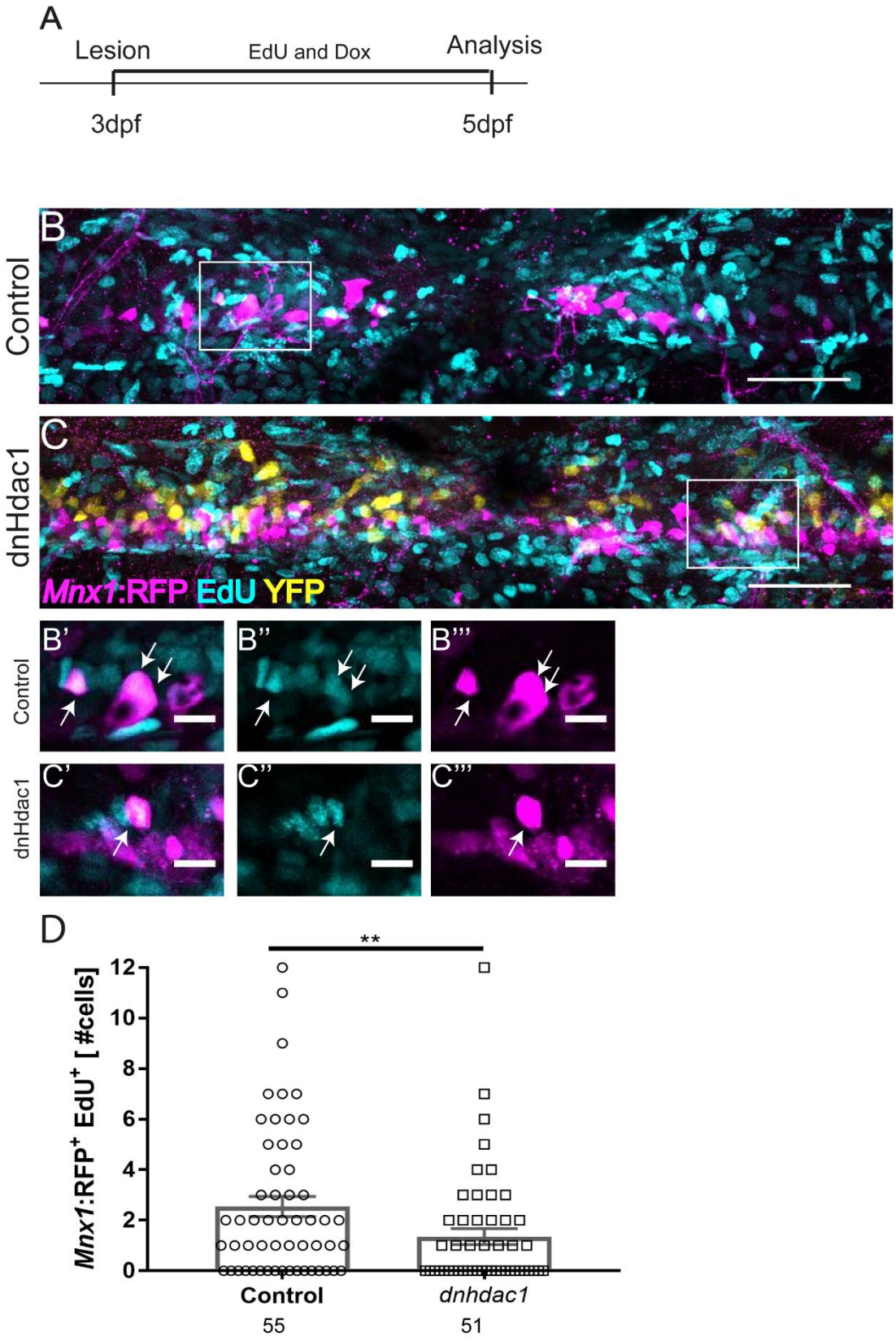

A
Lesion
EdU and Dox
Analysis
3dpf
5dpf
B
Control
C
dnHdac1
Mnx1:RFP EdU YFP
B'
B''
B'''
Control
C'
C''
C'''
dnHdac1
D
**
Mnx1:RFP+ EdU+ [#cells]
12
10
8
6
4
2
0
Control
55
dnhdac1
51

Figure 5-5: Expression of dnhdac1 in progenitors reduces number of new born motor neurons after injury. **A;** Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Mnx1*:RFP (magenta); *Her4.1*:TetA:TetRE:YFP-dnHdac1(yellow) larvae at 2dpl labelled with EdU (cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive *Mnx1*:RFP and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. Total N numbers are written below, each dot is a fish; 3 independent experiments. Mann-Whitney test, **P=0.0093, Post hoc power analysis: 0.63

Since the expression of *dnhdac1* was not restricted to the pMN ERGs I investigated whether the regeneration of other neuronal subtypes is affected. I used HuC immunohistochemistry to label all neurons in the lesioned spinal cord. This antibody recognises the Elav family members HuC, HuD and Hel-N1 neuronal proteins. It has been shown to specifically label neuronal cells in most vertebrate species. This would allow the study to observe if more than the motor neurons were affected by *dnhdac1* expression. The number of HuC/EdU double positive cells was assessed at 2 days post lesion. Comparable to the motor neuron counts, larvae expressing *dnhdac1* had a 43% reduction in the number of new born neurons after a lesion (Control 6.834±0.9285; dnHdac1 3.917±0.6017, Figure 5.6).

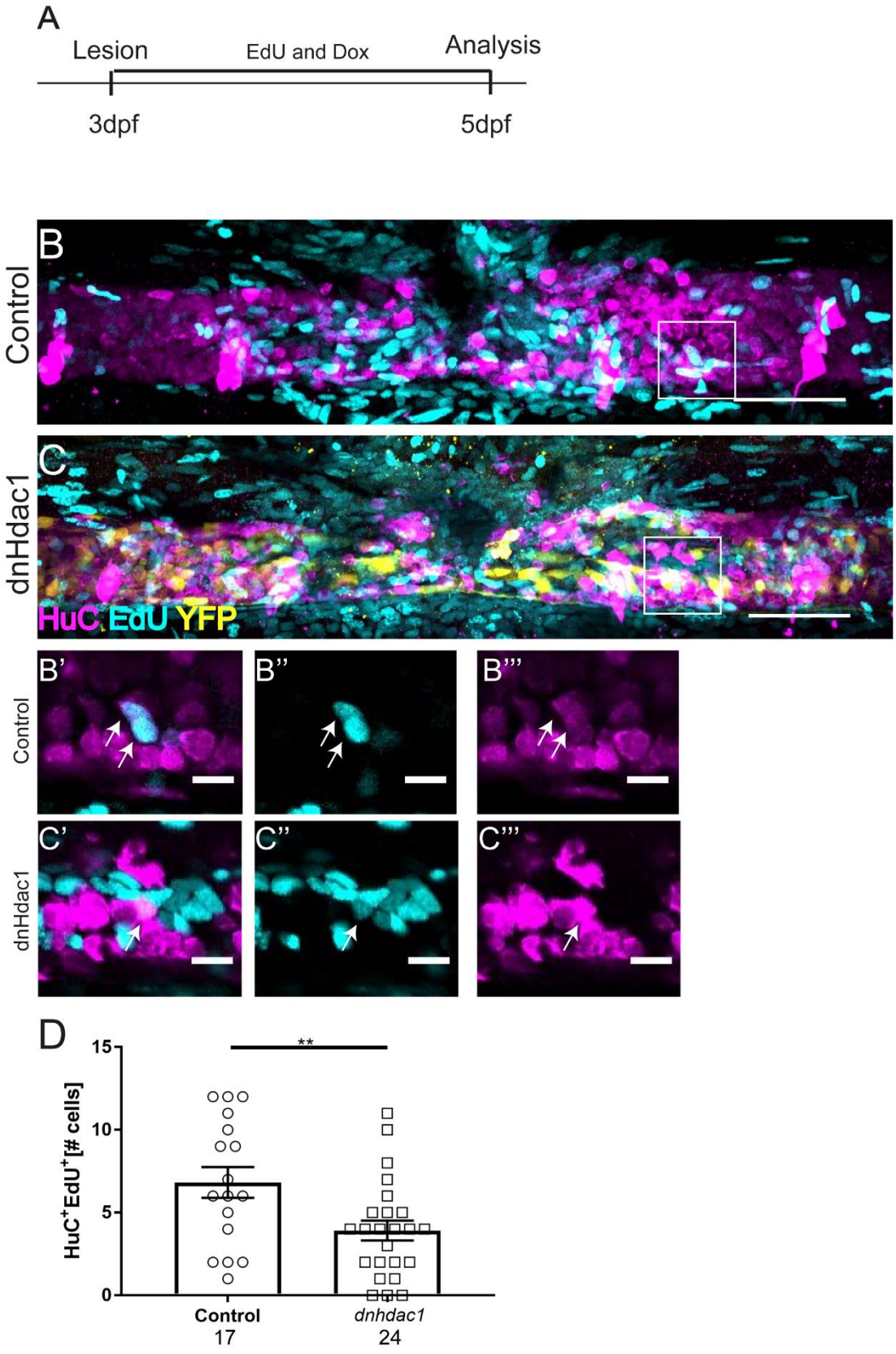

A
Lesion
EdU and Dox
Analysis
3dpf
5dpf
Control
dnHdac1
B
C
HuC EdU YFP
B'
B''
B'''
C'
C''
C'''
D
HuC+EdU+[# cells]
15
10
5
0
**
Control
17
dnhdac1
24

Figure 5-6: Expression of dnhdac1 in progenitors reduces numbers of new neurons after injury. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Her4.1*:TetA;TetRE:YFP-dnHdac1 (yellow) larvae at 2dpl labelled with anti-HuC (magenta) and EdU (cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive HuC and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. Total N numbers are written below, each dot is a fish; 1 independent experiment. Unpaired T-test, **P=0.0090, Post hoc power analysis: 0.744.

5.2.3 Cell specific expression of dnHdac1 reduces progenitor proliferation after a larval lesion

The reduction in the numbers of new born motor neurons after *dnhdac1* expression could be explained by three possible mechanisms. The lesion induced proliferation of progenitors is reduced, the progenitors fail to differentiate into neurons or the progenitors expressing the constructs die. To assess which process was occurring, double transgenic *Her4.1*:TetA;TetRE:YFP-dnHdac1 fish were crossed to *Olig2*:DsRed reporter fish (Kucenas *et al.*, 2008). This reporter line labels the pMN ERGs, motor neurons and oligodendrocytes with DsRed protein. From a previous study in the group (Ohnmacht *et al.*, 2016) it is known that after a lesion at 3dpf the pMN domain ERGs proliferate and stops producing oligodendrocytes. The rate of proliferation of the pMN domain can be measured by counting the number of double positive *Olig2*:DsRed/EdU cells at 1 day post lesion. The number of Olig2:DsRed/EdU double positive cells was shown to be increased by 165% after a lesion compared to the unlesioned spinal cord. A short EdU incubation window for 4 hours was used to label acutely proliferating cells. The animals expressing *dnhdac1* were found to have the same overall number of *Olig2*:DsRed cells proliferating after a lesion (Control 5.87±0.71419; *dnhdac1* 5.318±0.6818, Figure 5.7D).

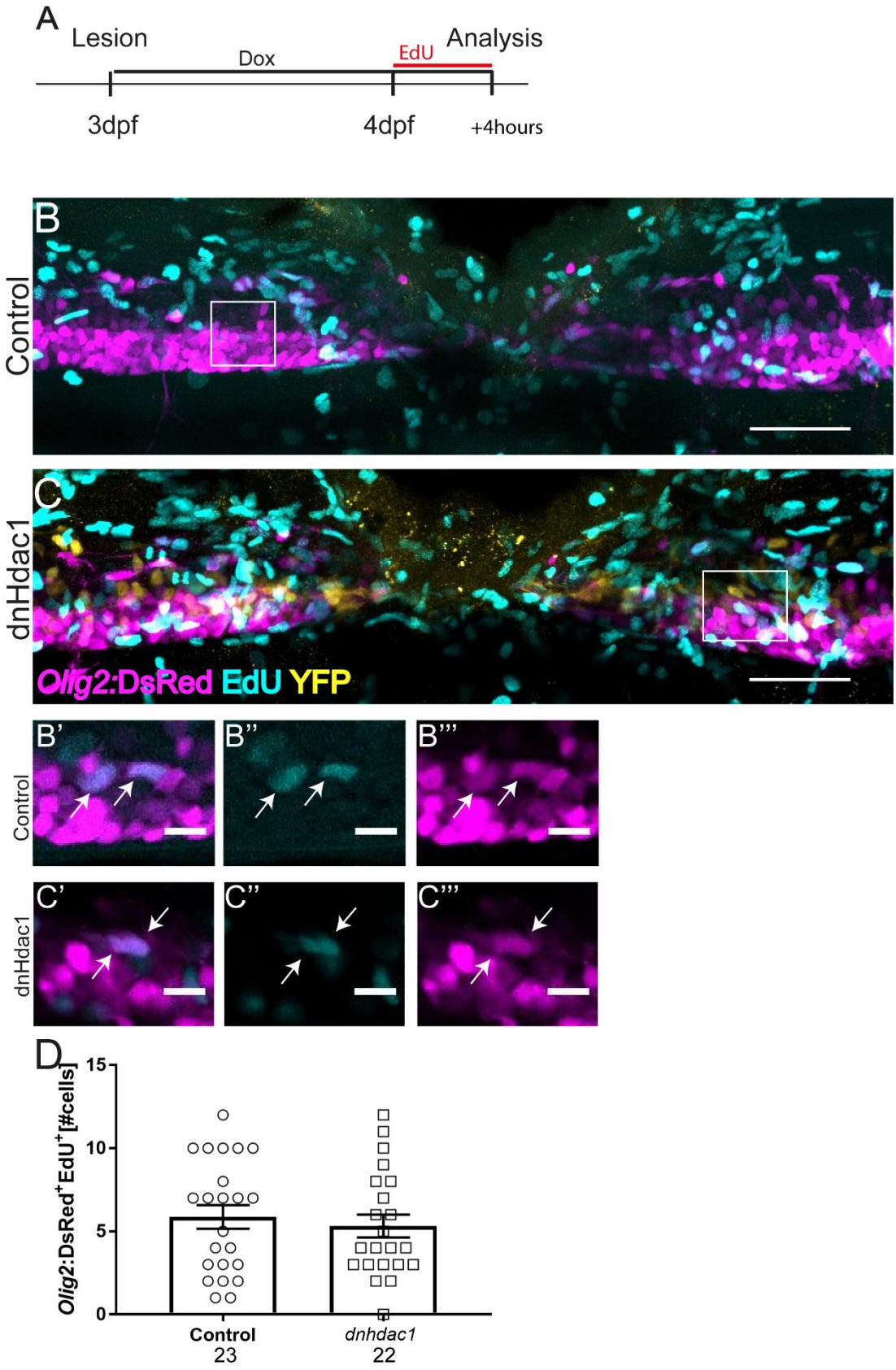
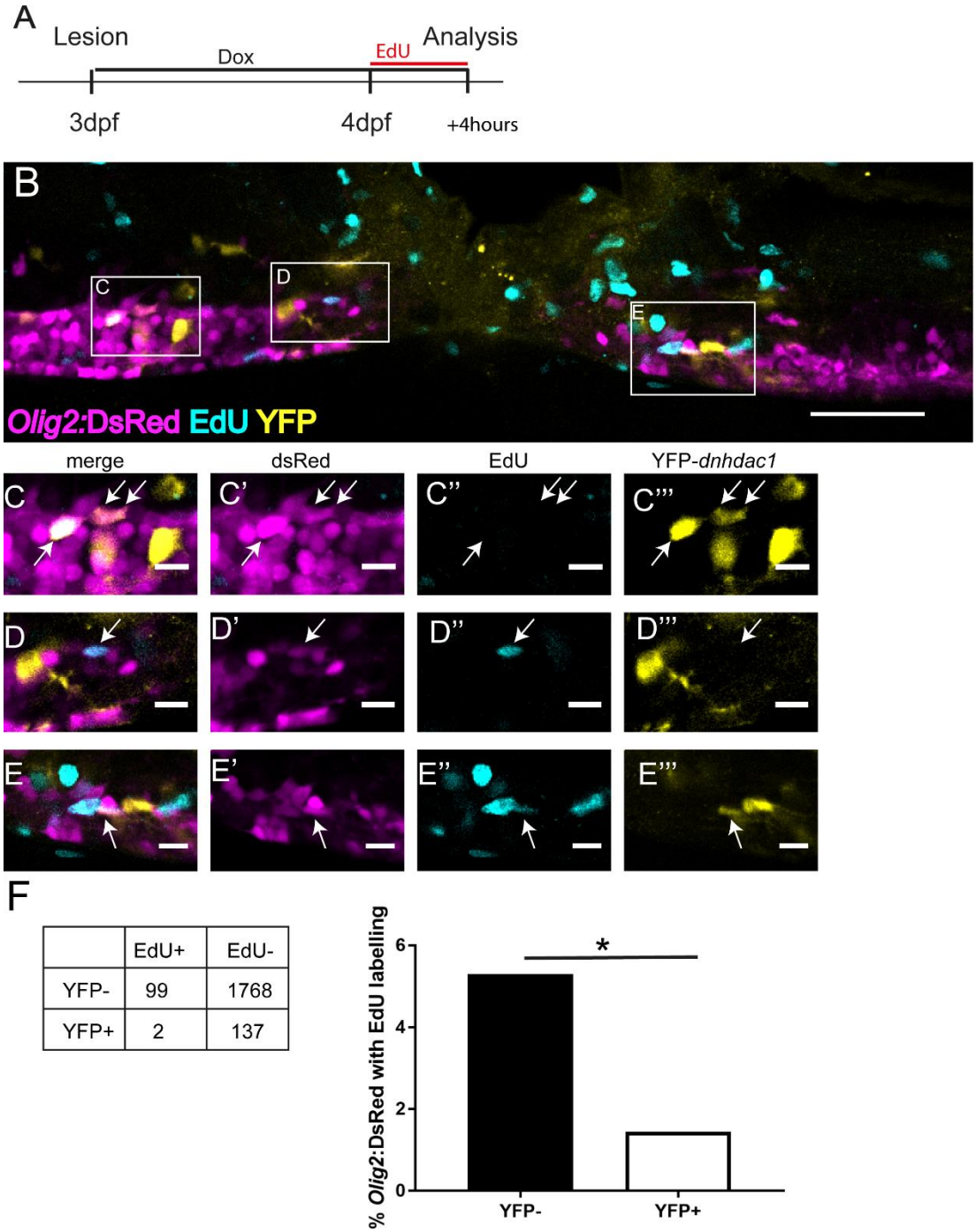

A
Lesion
Dox
EdU
Analysis
3dpf
4dpf
+4hours
B
Control
C
dnHdac1
Olig2:DsRed EdU YFP
B'
B''
B'''
Control
C'
C''
C'''
dnHdac1
D
Olig2:DsRed+EdU+[#cells]
15
10
5
0
Control
23
dnhdac1
22

Figure 5-7: Expression of dnhdac1 in progenitors did not affect overall lesioned induced proliferation of Olig2:DsRed cells. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Olig2*:DsRed (magenta); *Her4.1*:TetA;TetRE:YFP-dnHdac1 (yellow) at 2dpl labelled with EdU (cyan). Lateral views of injury site are shown; lesion site is centre. Scale bar is 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive *Olig2*:DsRed and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. Total N numbers are written below, each dot is a fish; 2 independent experiment. Unpaired T-test, P=0.5802, A priori power animals: 568 animals/group.

This assessment analysed the total number of pMN ERGs that were proliferating after a lesion. An effect on proliferation could be masked by *Olig2*$^{+}$ cells that did not express the transgene. After doxycycline induction 6.9% of the *Olig2*:DsRed cells around the lesion site expressed YFP-*dnhdac1* (as shown in Figure 5.8C) and the majority, 93.1%, remained negative for YFP-*dnhdac1* (as shown in Figure 5.8E). To more acutely measure proliferation, I separately counted the number of EdU positive cells within the *Olig2*:DsRed^{+}/YFP^{-} population (top row of table in Figure 5.8F) and the number of EdU positive cells within the *Olig2*:DsRed^{+}/YFP^{+} population (bottom row of table in Figure 5.8F). The percentage of the EdU positive cells between the two populations was then compared. The triple labelled cells were extremely rare (only 2 triple labelled cells found in 22 fish). The *Olig2*:DsRed cells that expressed *dnhdac1* showed a significant decreased proliferation rate (*Olig2*:DsRed^{+}/YFP^{-} 5.3%; *Olig2*:DsRed^{+}/YFP^{+} 1.44%, Figure 5.8F). Hence, proliferation of cells in the pMN domain after lesion is reduced by *dnhdac1* expression.



	EdU+	EdU-
YFP-	99	1768
YFP+	2	137

Figure 5-8: Expression of dnhdac1 in progenitors reduces proliferation of Olig2:DsRed cells after injury. A; Timeline of the experiment. **B**; Representative single optical section of *Olig2*:DsRed (magenta): *Her4.1*:TetA;TetRE:YFP-dnHdac1 (yellow) larvae at 1dpl labelled with EdU (cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm. **C-E'''**; Double or triple labelled cells from white squares of C,D,E in single optical sections. White arrows point to double or triple labelled cells. Scale bars are 10µm. **F**; Table of numbers of double and triple labelled cells in 250µm around the lesion site. Graph is the percentage of DsRed and EdU cells in both the YFP negative and YFP positive populations.

N=22; 2 independent experiments. Fishers exact test, *P=0.0428, Post hoc power analysis: 0.063.

5.2.4 Pharmacological inhibition of HATs has no effect on motor neuron regeneration

As the expression of a possible dominant negative Hdac1 was able to reduce regenerative neurogenesis in zebrafish larvae the study next tested to see if decreasing acetylation could affect regenerative neurogenesis. To do this *Hb9*:GFP larvae were lesioned and treated with the HAT inhibitor EML425 after injury. This reporter line labels the motor neurons with green fluorescent protein (Flanagan-Steet *et al.*, 2005). EML425 had been found to increase neurogenesis in the intact larval spinal cord (chapter 4) at 3µM so same concentration was used for experiments in the lesioned conditions. The numbers of double positive *Hb9*:GFP/EdU cells was assessed at 2 days post lesion. Treatment of EML425 at 3µM did not significantly increase the number of new born motor neurons after a lesion (Vehicle 11.61±1.771; EML425 14.67±2.044, Figure 5.9D).

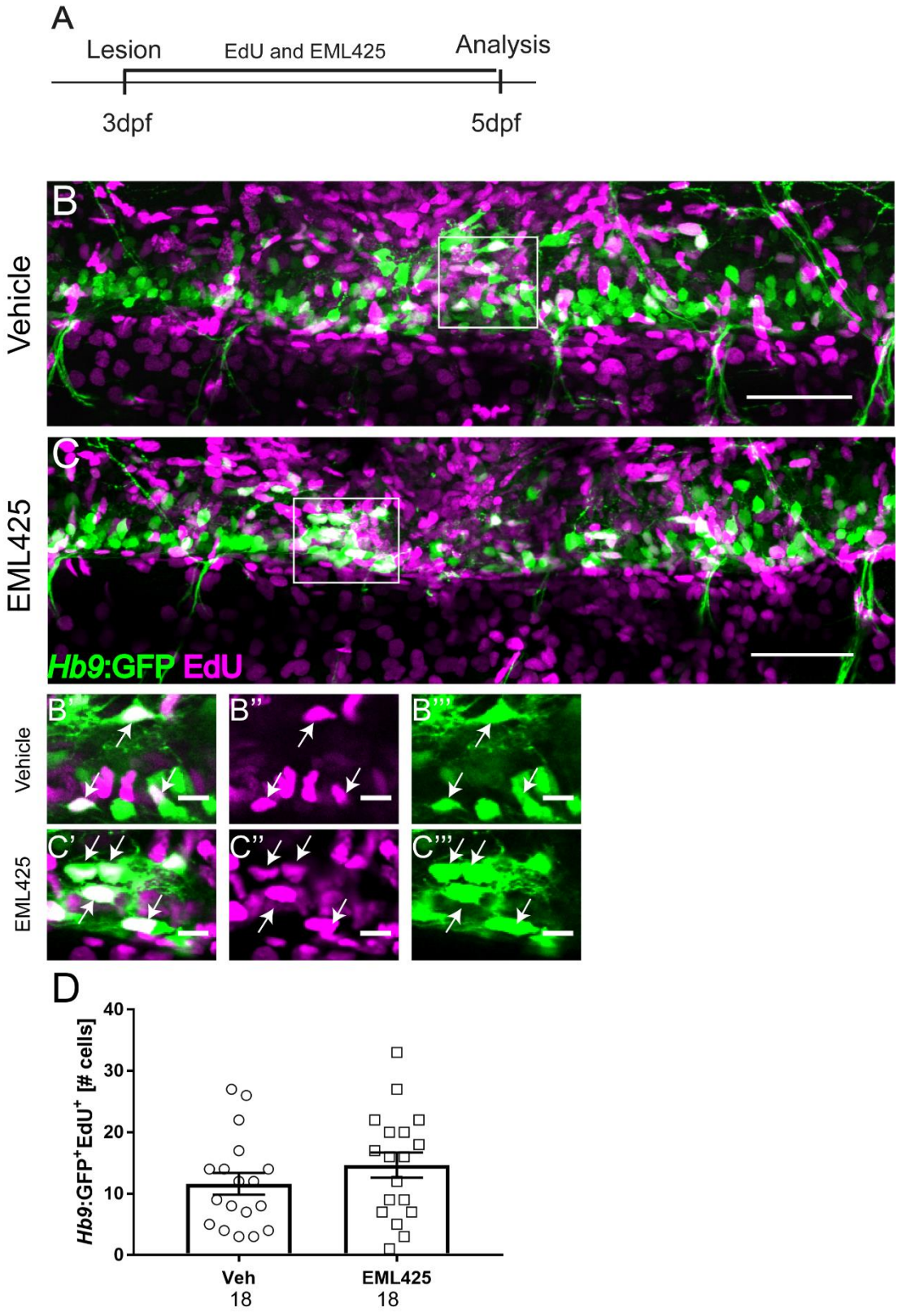


Figure 5-9: HAT inhibition does not affect the numbers of new motor neurons after injury. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Hb9*:GFP (green) larvae at 2dpl labelled with EdU (magenta) after treatment with vehicle or

3µM EML425. Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive *Hb9*:GFP and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. N numbers are written below, each dot is a fish; 2 independent experiments. Unpaired T-test, P=0.2665, A priori power analysis: 112 animals/group.

5.2.5 Cell specific overexpression of Hdac1 has no effect on motor neuron regeneration

Since HAT inhibition is not a direct method to test the effect of HDAC overexpression the new transgenic lines were used to increase Hdac1 expression in the progenitor after a lesion. The *Her4.1*:TetA;TetRE:YFP-Hdac1 double transgenic animals crossed to *Mnx1*:RFP reporter fish were lesioned and induced with doxycycline. The numbers of double positive *Mnx1*:RFP/EdU cells was assessed at 2 days post lesion. The larvae expressing *hdac1* in the ERGs showed no significant change in the number of new born motor neurons after a lesion (Control 1.774±0.4417; *Hdac1* 2.519±0.5453, Figure 5.10).

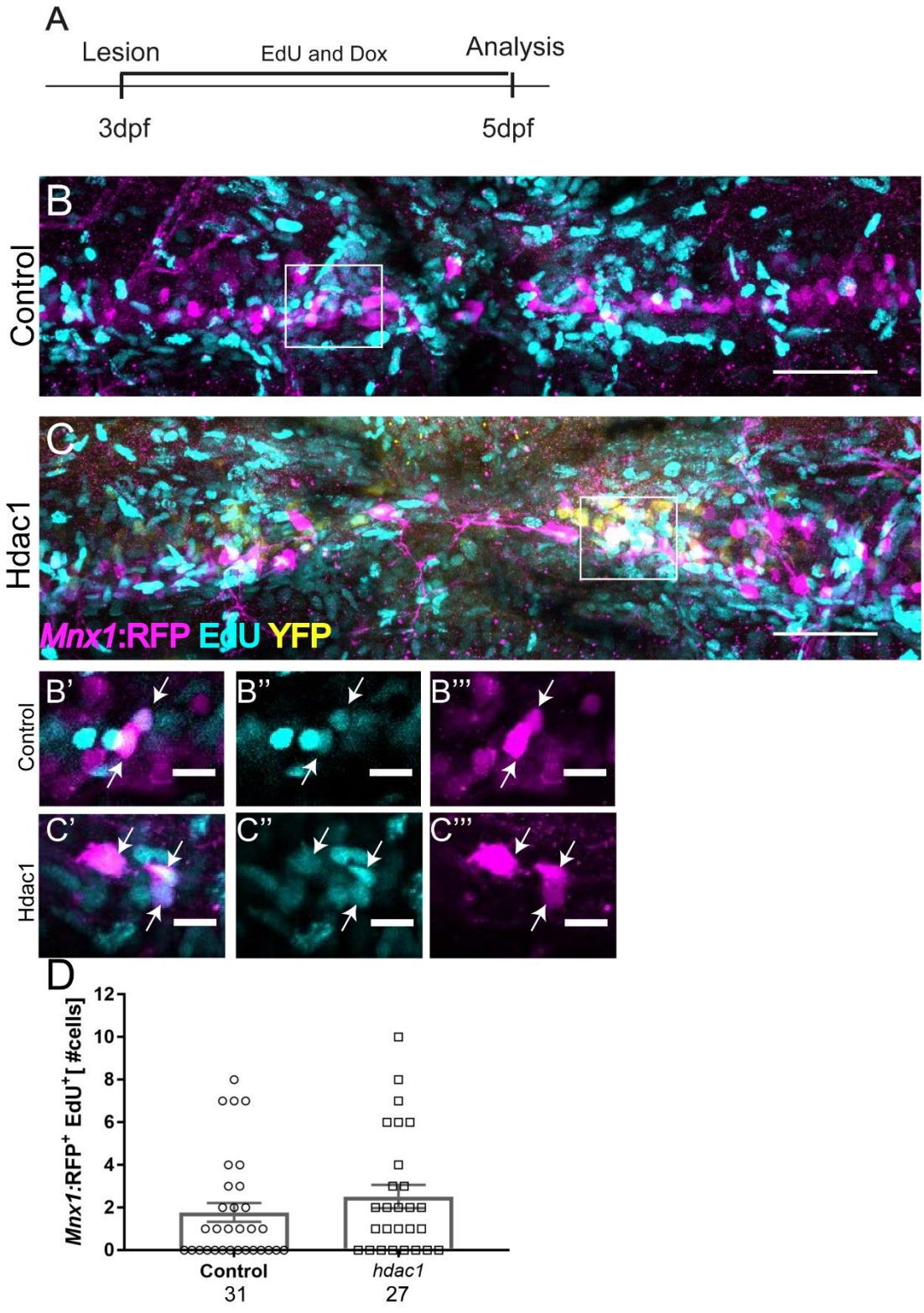


Figure 5-10: Expression of hdac1 in progenitors does not affect the number of new born motor neurons after injury. **A**; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Mnx1*:RFP (magenta) ;*Her4.1*:TetA;TetRE:YFP-Hdac1(yellow) larvae at 2dpl labelled with EdU (cyan).Lateral views of the injury are shown; lesion site is centre.

Scale bars are 50µm **B'-C'''**; Double labelled cells from white squares of B,C in single optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive *Mnx1*:RFP and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. N numbers are written below, each dot is a fish; 3 independent experiments. Mann-Whitney, P=0.229, A priori power analysis: 200 animals/group.

I next tested whether *hdac1* overexpression had a global effect on neurogenesis that could be missed using only a motor neuron marker. HuC immunohistochemistry was used to label all neurons. The number of HuC/EdU double positive cells was assessed at 2 days post lesion. In line with the motor neuron assessment, larvae expressing *hdac1* showed no significant change in the number of new born neurons after a lesion (Control 16±1.535; Hdac1 19.33±2.64, Figure 5.11).

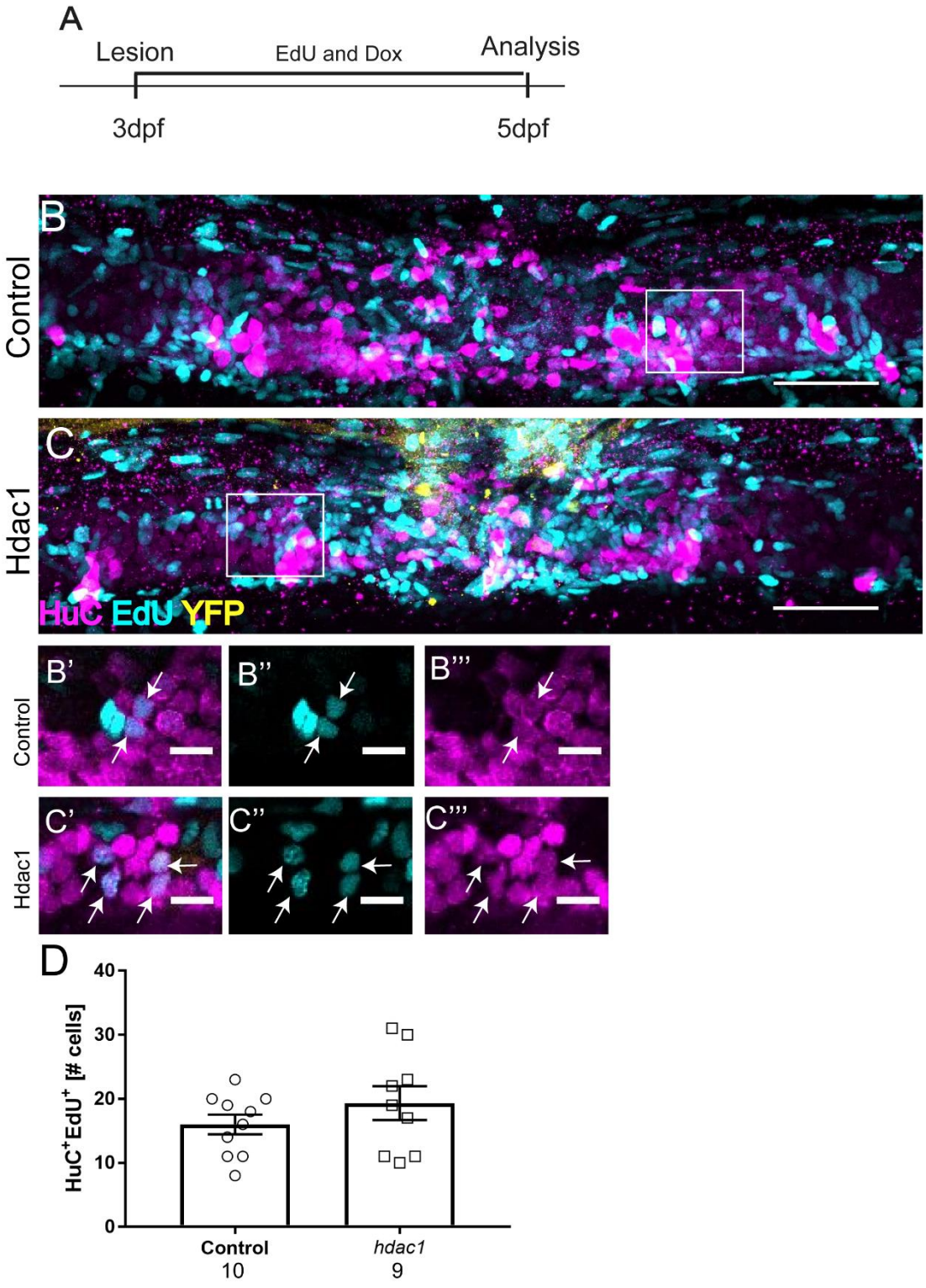


Figure 5-11: Expression of hdac1 in progenitors does not change the numbers of new neurons after injury. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Her4.1*:TetA;TetRE:YFP-Hdac1 (yellow) larvae at 2dpl labelled with anti-HuC (magenta) and EdU (cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single optical

sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive HuC and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as bar and scatter plot. N numbers are written below, each dot is a fish; 1 independent experiment. Unpaired T-test, P=0.2785, A priori power analysis: 63 animals/group.

5.2.6 Cell specific overexpression of Hdac1 may reduce progenitor proliferation after injury

No change in neuroregeneration was observed after *hdac1* overexpression but *hdac1* may still have affected the behaviour of the progenitors after the lesion. To assess this, the level of lesion induced proliferation of the progenitors after *hdac1* overexpression was investigated. The *Olig2*:DsRed reporter line and the 4 hour incubation with EdU at 1 day post lesion was used to quantify the proliferation of the pMN progenitor cells. Animals expressing *hdac1* had a significant decrease in the overall number of *Olig2*:DsRed cells proliferating after a lesion (Control 6.273±1.096; hdac1 3.222±0.8784, Figure 5.12)

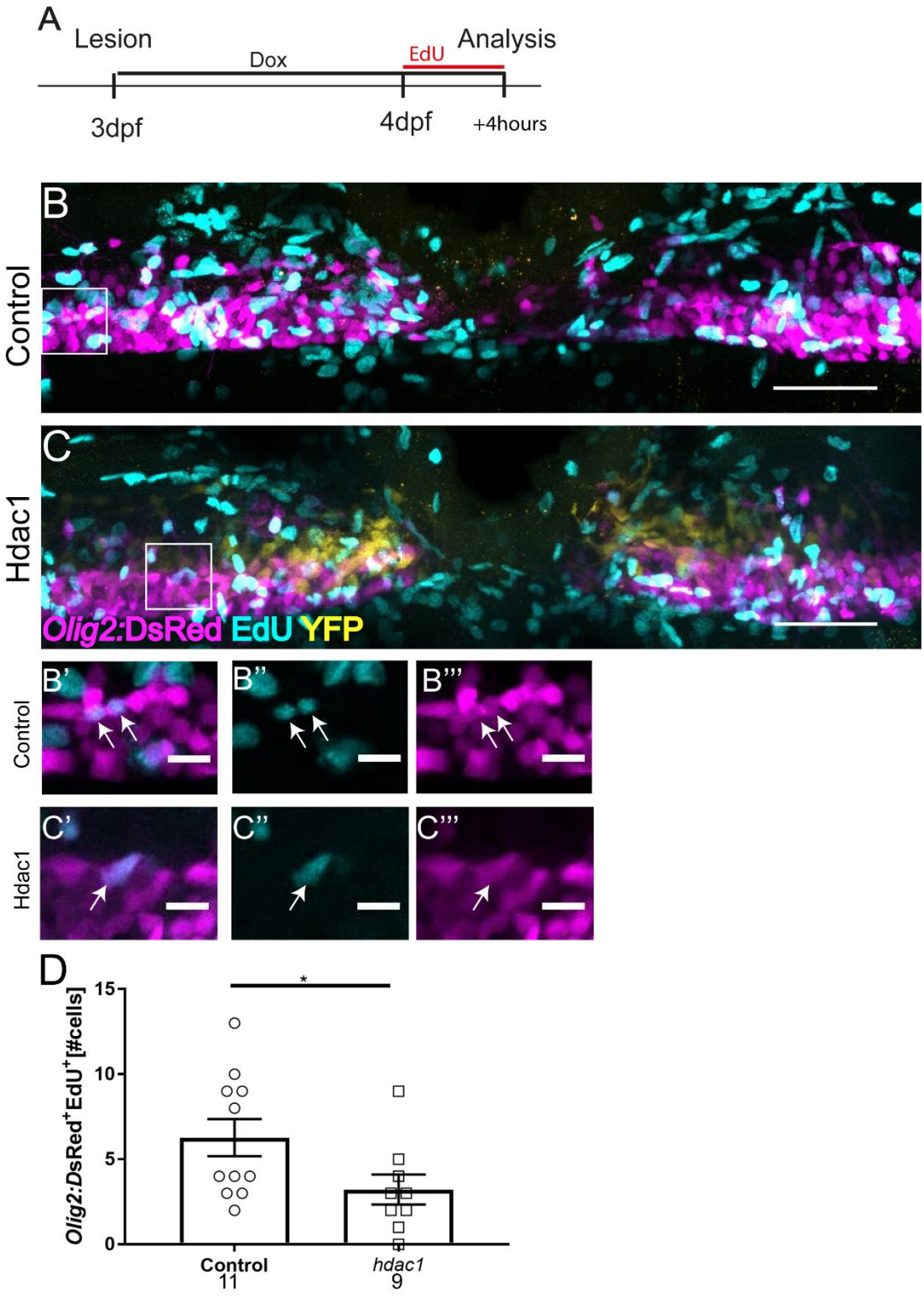


Figure 5-12: Expression of hdac1 in progenitors decreases the overall lesion induced proliferation of Olig2:DsRed cells. A; Timeline of the experiment. **B-C**; Representative maximal intensity projections of *Olig2*:DsRed (magenta); Her4.1:TetA;TetRE:YFP-dnHdac1 (yellow) at 2dpl labelled with EdU (cyan). Lateral views of injury site are shown; lesion site is centre. Scale bar is 50µm. **B'-C'''**; Double labelled cells from white squares of B,C in single

optical sections. White arrows point to double labelled cells. Scale bars are 10µm. **D**; Quantification of the number of double positive *Olig2*:DsRed and EdU cells in 250µm around the lesion site. Data shown as mean±SEM as a bar and scatter plot. Total N numbers are written below, each dot is a fish; 1 independent experiment. Unpaired T-test, P=0.0499, Post hoc power analysis:0.525.

Similarly to the induced *dnhdac1* expression, I found that after doxycycline induction 14.6% of the *Olig2*:dsRed cells expressed YFP-*hdac1* and the majority, 85.4%, remained negative for the transgene. To assess proliferation more precisely as performed after *dnhdac1* expression, I took advantage of the mosaicism of the YFP expression within the *Olig2*:DsRed population. I counted the number of EdU positive cells within the *Olig2*:DsRed^{+}/YFP^{-} (white arrows in Figure 5.13B''-B''') population and compared it to the number of EdU positive cells within the double positive *Olig2*:DsRed^{+}/YFP^{+} population. Triple labelled cells were very rare (only 1 in 9 fish) and nearly all the *Olig2*:DsRed^{+}/YFP^{+} cells were negative for EdU (white arrowheads in Figure 5.13B'''). The percentage of EdU positive cells was compared between the two populations. The *Olig2*:dsRed cells that expressed *hdac1* showed a ~4-fold decreased rate of proliferation, which was not statistically significant (*Olig2*:dsRed^{+}/YFP^{-} 4.01%; *Olig2*:DsRed^{+}/YFP^{+} 0.87%, Figure 5.13).

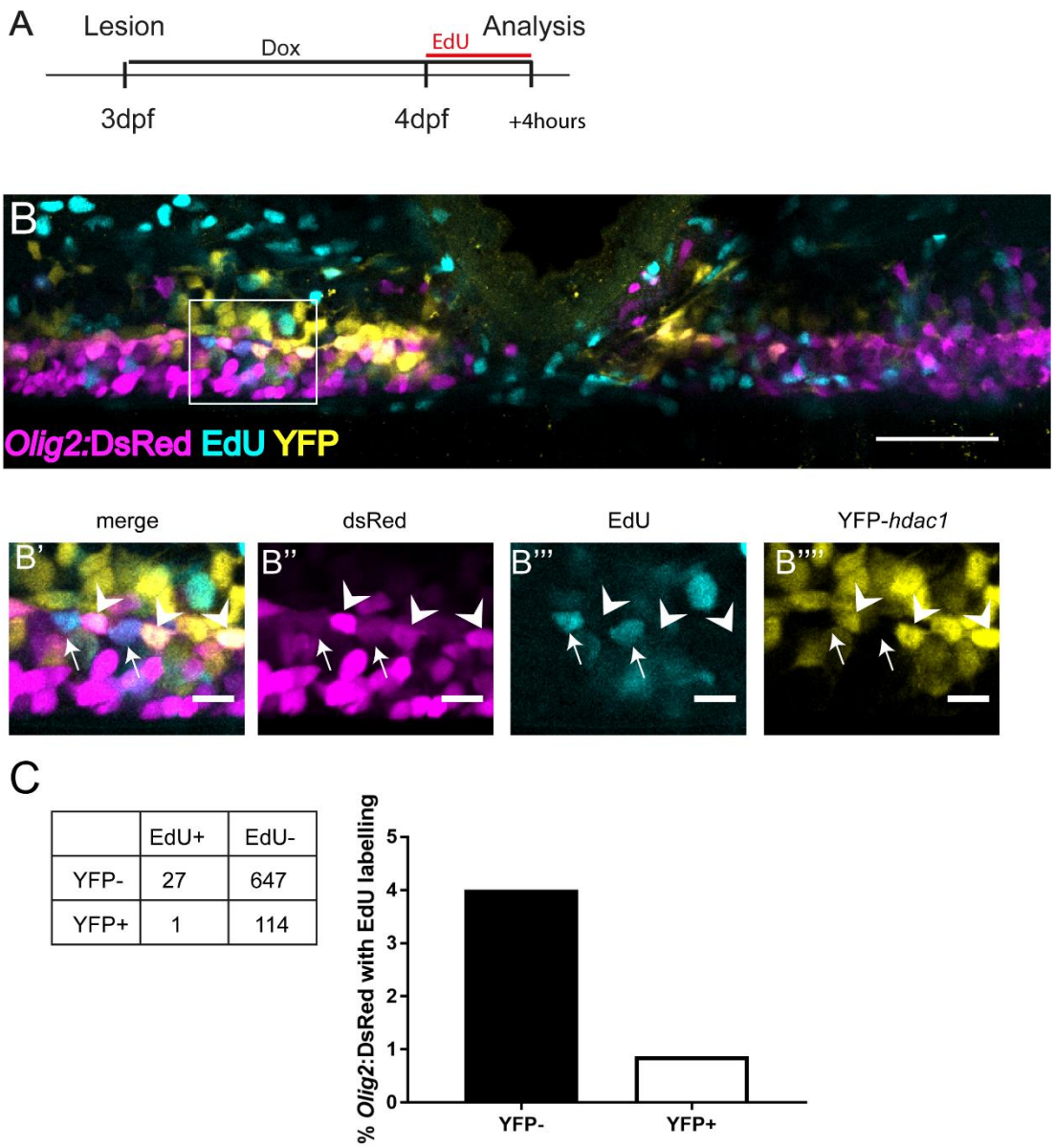


Figure 5-13: Expression of hdac1 in progenitors trends towards reducing the proliferation of Olig2:DsRed cells after injury. A; Timeline of the experiment. **B-C**; Representative single optical section of *Olig2*:DsRed(magenta); *Her4.1*:TetA;TetRE:YFP-Hdac1(yellow) larvae at 1dpl labelled with EdU(cyan). Lateral views of the injury are shown; lesion site is centre. Scale bars are 50µm. **B'-B'''**; Double labelled cells from white square of B in single optical sections. White arrows point to double labelled *Olig2*:DsRed^{+}/YFP^{-}/EdU^{+} cells while white arrowheads point to *Olig2*:DsRed^{+}/YFP^{+}/EdU^{-}. Scale bars are 10µm. **C**; Table of numbers of double and triple labelled cells in 250µm around lesion site. Graph is the percentage of *Olig2*:DsRed and EdU cells in both the YFP negative and YFP positive populations. N=9; 1 independent experiment. Fishers test, P=0.1613, A priori power analysis: 1306 YFP- and 222 YFP+.

As another method to measure progenitor proliferation after a lesion the pH3 immunohistochemistry as used in the unlesioned spinal cord was performed. Double transgenic *Her4:1*:TetA;TetRE:YFP-Hdac1 larvae were lesioned at 3dpf and induced with doxycycline. Animals were fixed at 1 and 2 days post lesion and processed. The AmCyan tag of the Tet activator line was used to label the progenitors. The larvae were imaged and the number of AmCyan positive cells that were pH3 positive was assessed. When looking at numbers of pH3 in relation to unlesioned values (shown in Chapter 4) there was only a significant increase in mitosis seen at 2 days post lesion (unlesion 0.2±0.106, 2dpl 2.5±0.4214; $P<0.0001$). However, at both time points after lesion larvae expressing *hdac1* had no change in the numbers of progenitors in mitosis compared to controls (1dpl: Control 1.05±0.3202, hdac1 0.8±0.2128; 2dpl: Control 2.5±0.4212, hdac1 2.3±0.3632, Figure 5.14).

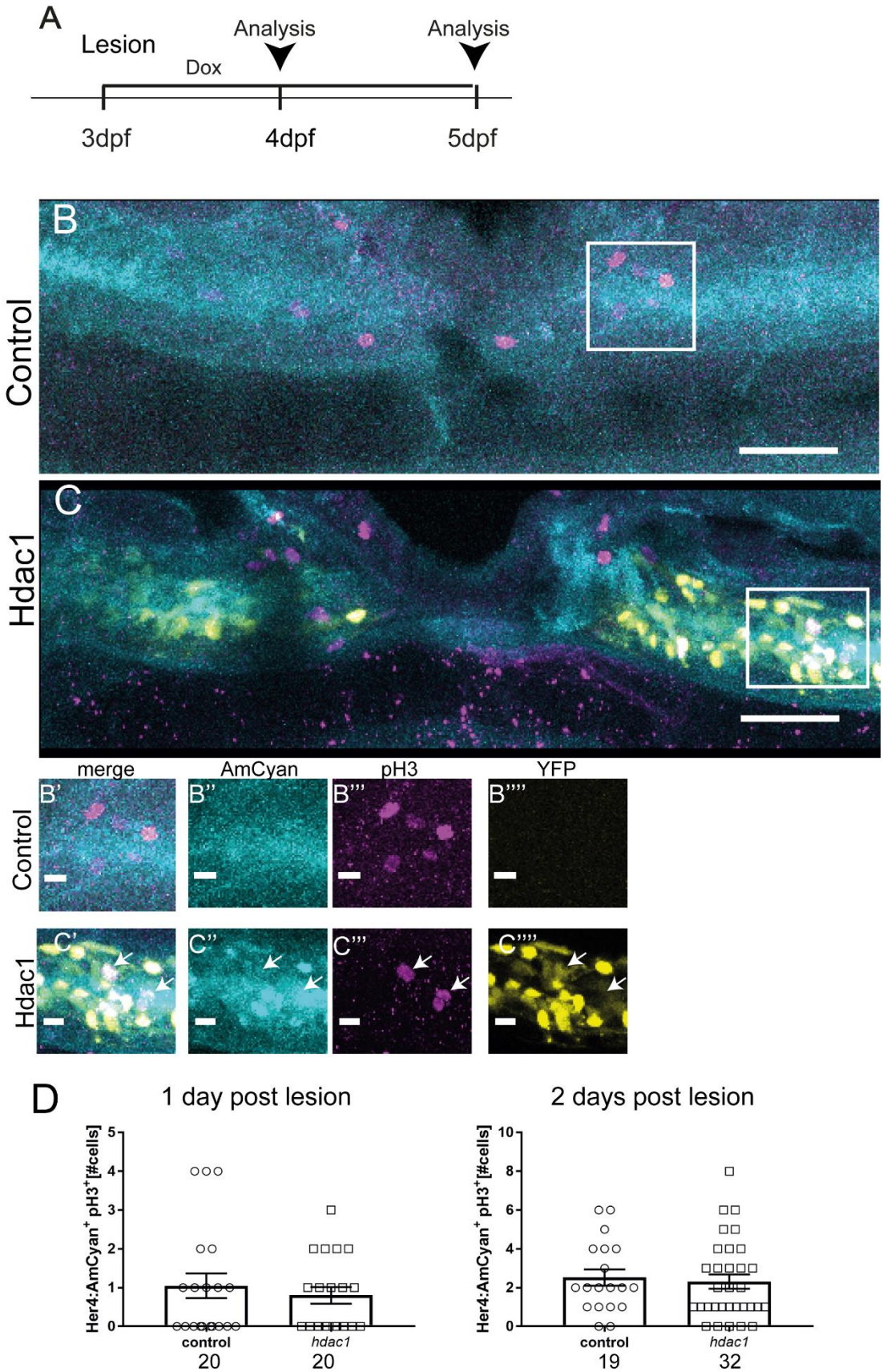

A
Lesion
Dox
Analysis
Analysis
3dpf
4dpf
5dpf
B
Control
C
Hdac1
merge
AmCyan
pH3
YFP
B'
B''
B'''
B''''
Control
C'
C''
C'''
C''''
Hdac1
D
1 day post lesion
2 days post lesion
Her4:AmCyan+ pH3+[#cells]
control
hdac1
20
20
19
32

Figure 5-14: Expression of hdac1 does not change the number of progenitors in mitosis after injury. **A**; timeline of experiment. **B-C**: Representative maximal intensity projections of *Her4.1*:TetA;TetRE:YFP-Hdac1 larvae labelled with pH3. Scale bar is 50µm. **B'-C'''**: Close up of double labelled cells from white squares in B,C. white arrows point to double labelled cells. Scale bar is 10µm. **D**: Quantification of the number of double positive *Her4.1*:AmCyan and ph3 cells in 250µm around the lesion site at 1 day and 2 day post lesion. Data shown as mean±SEM as a bar and scatter plot. Total N numbers are written below, each dot is a fish; 3 independent experiments. Unpaired t-test, A priori power analysis: 373 animals/group; 1169 animals/group.

5.2.7 Cell specific manipulation of Hdac1 after adult lesion had no effect on neuroregeneration or proliferation

There are similarities between the processes of larval and adult spinal cord regeneration (Ohnmacht *et al.*, 2016). To investigate if Hdac1 activity was also necessary or sufficient in adult regeneration I used adult double transgenic animals to overexpress *dnhdac1* and *hdac1* in ERGs after a lesion to the spinal cord. Double transgenic animals were treated with doxycycline immediately after lesion until 14dpl, when motor neuroregeneration is at its peak (Reimer *et al.*, 2008). The number of small Hb9 positive cells in the lesioned spinal cord cross sections was determined with stereological counting. Previous studies by the group (Reimer *et al.*, 2008) found that these cells co-label with BrdU indicating they are newly born after the lesion and are very rarely found in the unlesioned spinal cord. In WIK animals treated with doxycycline clusters of intensely labelled small Hb9 positive cells were observed in the ventro-medial aspect of spinal cord cross sections. The numbers of these Hb9 positive cells were found to be comparable to previous observations (Reimer 2008; 870±106.8 cells). This further demonstrates that the doxycycline treatment regime per se did not influence motor neuron regeneration.

In animals expressing *dnhdac1* or *hdac1* in spinal cord progenitors the number of Hb9 positive cells did not significantly change in the total 1500µm around the lesion site (Total: WIK 776.3±130.6, *dnhdac1* 732±79.47, *hdac1* 762.5±105.3, Figure 5.15C). Other manipulations, such as the monoaminergic transmitters dopamine and serotonin, had specific effects on neuroregeneration depending on location to the lesion site. Therefore, I examined the number of Hb9 positive cells in either 750µm rostral or caudal to the lesion site after the Hdac1 manipulations. After a lesion, there is a rostral-caudal asymmetry with more Hb9 positive cells generated on the rostral side of the spinal cord (Barreiro-Iglesias *et al.*, 2015). In animals expressing *dnhdac1* or *hdac1* no significant difference was found compared to control animals. (Rostral: WIK 563±116.4, *dnhdac1* 487±80.08, *hdac1* 590.4±116.6; Caudal: WIK 210±14.96, *dnhdac1* 244.4± 79.21, *hdac1* 172.1± 64.99, Figure 5.15D).

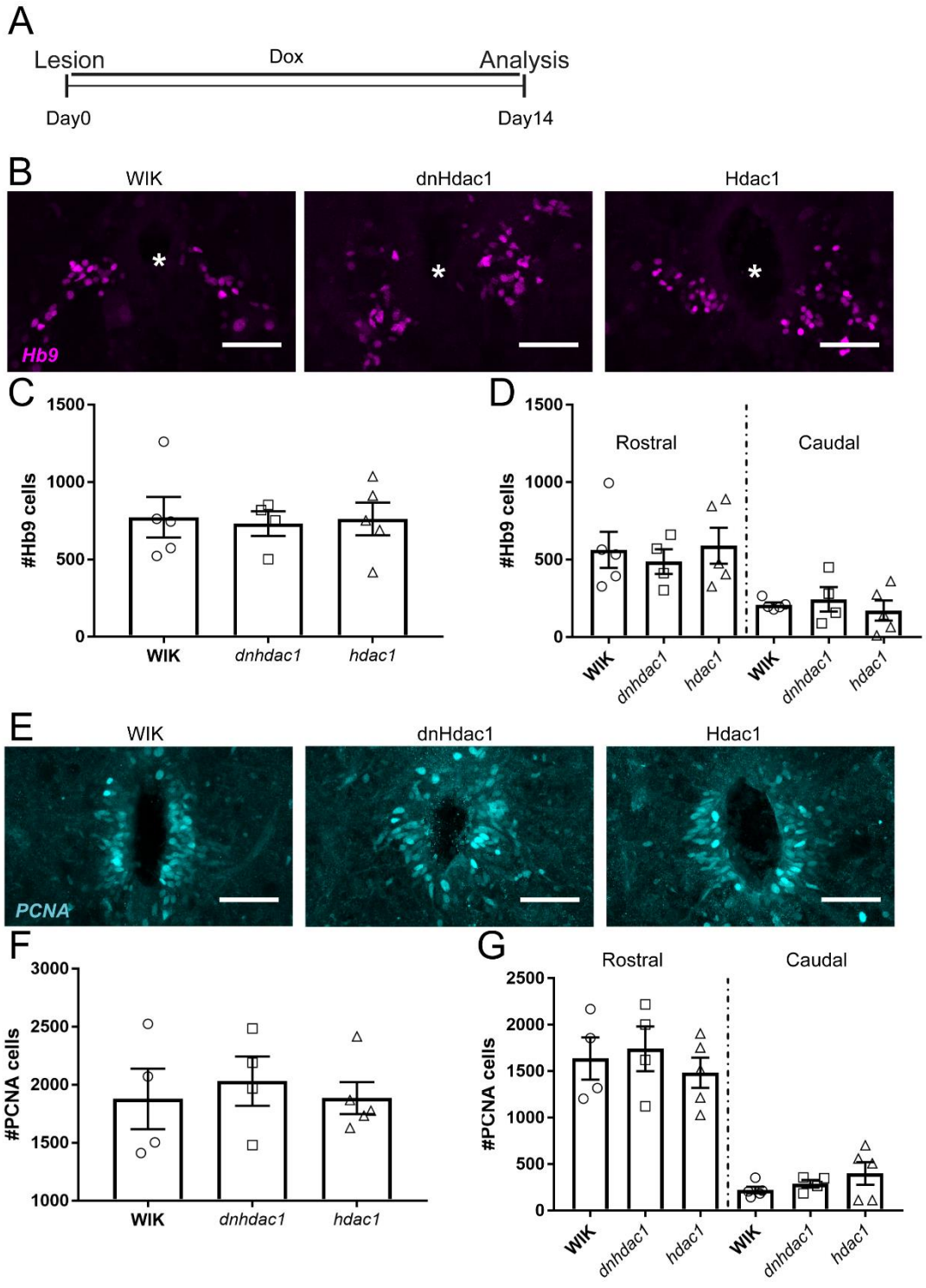


Figure 5-15: Expression of dnhdac1 or hdac1 in progenitors does not affect motor neuron regeneration or ventricular proliferation in the lesioned adult spinal cord. A; Timeline of experiment. **B**; Representative spinal cord sections centred around the central canal labelled with anti-Hb9 (magenta), dorsal is up. Asterisk marks the central canal. **C-D**; Quantification of number of Hb9-positive cells; C is total counts and D is split depending on location to lesion site. Data shown as mean±SEM as bar and scatter plot. Each dot is a fish. One-way ANOVA, Tukeys multiple comparison, all P>0.05. **E**; Representative spinal cord

sections centred around the central canal labelled with anti-PCNA (cyan), dorsal is up. **F-G**; Quantification of number of ventricular PCNA-positive cells; F is total counts and G is split depending on location to lesion site. Data shown as mean±SEM as a bar and scatter plot. Each dot is fish. 1 independent experiment. One-way ANOVA, Tukeys multiple comparison, all P>0.05.

To see if the expression of *dnhdac1* or *hdac1* in the ERGs effected ventricular proliferation after a lesion I used immunohistochemistry for Proliferating cell nuclear antigen (PCNA). PCNA labels all cells in early G1 phase and S phase of the cell cycle. PCNA positive cells are mostly found in the ventricular zone and increase in number after a lesion as previously reported (Reimer *et al.*, 2008). In animals expressing *dnhdac1* or *hdac1* the total numbers of ventricular PCNA positive cells did not significantly change (Total: WIK 1879±260.6, *dnhdac1* 2032±212.1, *hdac1* 1885±138.4, Figure 5.15F). The numbers of PCNA positive cells remained not significantly different after counts were split according to location to the lesion site (Rostral: WIK 1638±227.3, *dnhdac1* 1742±240.4, *hdac1* 1485±162.8; Caudal: WIK 221.6±35.78, *dnhdac1* 289.5±39.83, *hdac1* 400.4±121.4, Figure 5.15G).

5.2.8 Preliminary investigations into the link between the immune system and Hdac1 activity

The immune system is necessary for neuroregeneration in the zebrafish brain (Kyritsis *et al.*, 2012) and spinal cord (Tsarouchas *et al.*, 2018). The downstream molecular mechanisms of how the immune system regulates the regenerative neurogenesis is unknown. Immune system signals have been found to stimulate changes in Hdac1 expression in other cell types (Bartl *et al.*, 1997) . The new transgenic lines allow separate manipulations of the immune system and Hdac1 activity in ERGs to be combined to investigate this possible connection. Double transgenic animals were crossed to *Mnx1*:RFP, lesioned and induced at 3dpf. They were additionally treated with two different pharmacological compounds that regulate the immune system; Lipopolysaccharide (LPS) or TNF-α inhibitor Pomalidomide. LPS can stimulate the immune response in mammalian models (Singh and Jiang, 2003) and can increase the number of macrophages at the lesion site of larval zebrafish spinal cord (Tsarouchas *et al.*, 2018). LPS treatment increased the number of new born motor neurons after a lesion in larval zebrafish (unpublished observations). TNF-α is a cytokine released by immune cells and can regulate regenerative neurogenesis in the zebrafish retina (Nelson *et al.*, 2013). Pomalidomide blocks the release of TNF-α and

reduced the number of new born motor neurons after a lesion in larval zebrafish (unpublished observations).

The *Her4.1*:TetA;TetRE:YFP-dnHdac1 line was used to test whether expression of *dnhdac1* in the ERG could block the increase in regenerative neurogenesis induced by LPS treatment. Separately, the *Her4.1*:TetA:TeRE:YFP-Hdac1 was used to test whether *hdac1* expression in the ERG could rescue the decrease in regenerative neurogenesis caused by pomalidomide treatment. The number of double labelled *Mnx1*:RFP/EdU cells was assessed at 2days post lesion as before. *Dnhdac1* or *Hdac1* expression did not change the number of new born motor neurons after a lesion compared to LPS or Pomalidomide respectively (Control 0.8±0.489; LPS 3.333±0.333; LPS*dnhdac1* 3.2±0.6633, Figure 5.16A) (Control 3.714±0.7143; POM 1.778±0.6186; POM*hdac1* 1.25±0.25, Figure 5.16B).

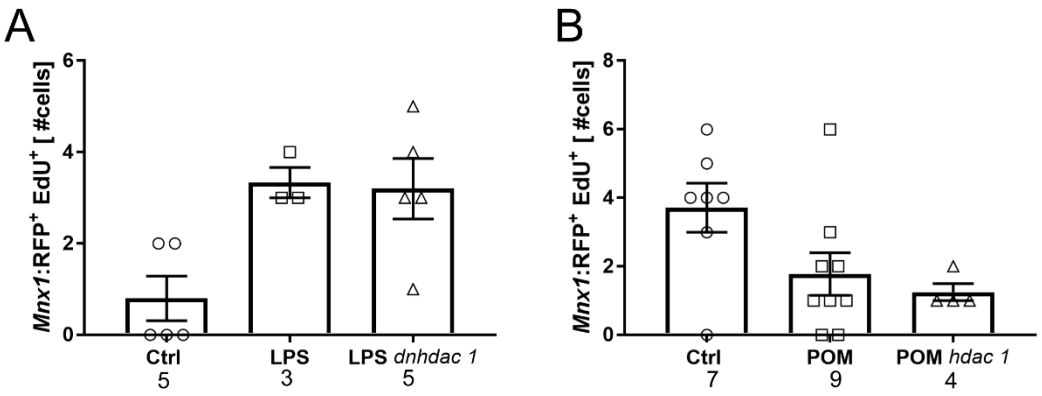


Figure 5-16: Preliminary experiments did not show an interaction between immune system and Hdac1. A-B; Quantification of double positive *Mnx1*:RFP and EdU cells in 250µm around the lesion site. *Mnx1*:RFP;Her4.1:TetA;TetRE:YFP-dnHdac1 were treated with LPS after lesion (A) and *Mnx1*:RFP;Her4.1:TetA;TetRE:YFP-Hdac1 were treated with Pomalidomide (POM) after lesion (B). Data shown as mean±SEM as bar and scatter plot. Total N numbers are written below, each dot is a fish; 1 independent experiments. Krustal-Wallis test, all P>0.05.

5.3 Discussion

In this chapter I used the newly generated transgenic lines to investigate the cell autonomous role of Hdac1 in the spinal cord ERGs in neuroregeneration.

Table 5-1: Summary of results of Chapter 5. Results for different manipulations are separated by lesion paradigm and cellular readout. ↑ significant increase, ↓ significant decrease, ─ no change and blank space experiment not performed in this study.

		dnHdac1	Hdac1	EML425
Larval lesion	Motor neurons	↓	─	─
	Total neurons	↓	─ trending increase	
	Total pMN proliferation	─	↓	
	Recombined pMN proliferation	↓	─	
Adult lesion	Motor neurons	─	─	
	Total ERG proliferation	─	─	

5.3.1 Tet-On system is suitable for use in neuroregeneration studies in zebrafish spinal cord

The Tet-On system had been used to manipulate regeneration in the caudal fin of zebrafish and doxycycline did not impede regeneration compared to vehicle in this context (Wehner *et al.*, 2014). No studies have used the system after a spinal cord lesion. I showed that the doxycycline concentration that induces transgene expression with the Tet-On system did not have any effects on the regeneration process after a larval lesion. Doxycycline did not negatively affect the immune response, as measured by the numbers of macrophages recruited to the lesion site. In fact, doxycycline treatment trended towards increasing the numbers of macrophages and A priori power analysis showed that with double the number of fish it would be statistically significant. Doxycycline treatment may influence the phenotype of the immune cells and not their recruitment to the injury. Therefore, additional experiments investigating cytokine levels could be examined to confirm that doxycycline does not alter the immune system. Doxycycline did not affect the numbers of new born motor neurons after a larval lesion or have any noticeable effect on neuroregeneration in adult zebrafish, as the number of new born motor neurons after a lesion by Hb9 immunolabelling was similar to what was observed previously (Reimer *et al.*, 2008). Therefore, the subsequent effects I see with the transgenic lines are due to expression of the different *hdac1* forms and not an indirect effect from the doxycycline treatment.

The Tet-On system provides a new tool for the study of molecular pathways in neuroregeneration in the zebrafish spinal cord. Additional pathways can be investigated using the activator lines, described in this study, combined with different responder lines. For example, the role of RA in neuroregeneration has not been functionally tested. RA can modulate the immune system (Larange and Cheroutre, 2016) so an ERG specific manipulation that is possible with the Tet-On system is required.

5.3.2 DnHdac1 expression reduced regenerative neurogenesis

The mechanism of action of dnHdac1 is still to be determined, however, expression of *dnhdac1* in the ERGs led to a reduction in the numbers of new born neurons after injury. This was observed using both motor neuronal and pan-neuronal markers. This supports the result obtained with the HDAC inhibitors. Both TSA and Mocetinostat reduced the numbers of new born motor neurons after a lesion. With this new result however, we have confirmed that it is Hdac1 within the progenitor that is necessary for the successful regenerative neurogenesis to occur. Therefore, Hdac1 has a positive role in regeneration in the zebrafish spinal cord. This is in contrast to previous studies in the mammalian spinal cord that found that reducing Hdac1 activity was beneficial for repair (Lv *et al.*, 2011; Bang *et al.*, 2013; Chu *et al.*, 2015). The cell specific manipulation of this study may be the reason for the difference observed.

Hdac1 can function as a positive (Cunliffe, 2004; Tao *et al.*, 2015) or negative (Stadler *et al.*, 2005; Yamaguchi *et al.*, 2005) regulator of proliferation of progenitor cells in the CNS depending on their location. No study had investigated the effect of Hdac1 on proliferation in the spinal cord ERGs after injury. I found that the expression of *dnhdac1* in pMN ERGs inhibited the lesion induced proliferation of these cells. This reduction in proliferation could be responsible for the subsequent reduction in new born motor neurons. Interestingly, the total number of proliferating pMN cells was not observed to be changed, highlighting the importance of cell specific analysis that can be performed with these transgenic lines. Analysis of the proliferation of the entire ERG population, not just the pMN domain, would provide further information on the effect of manipulating Hdac1 levels on regenerative proliferation. The *her4.1* transgene targets *dnhdac1* expression to more progenitor domains than the pMN domain. Additionally, *dnhdac1* expression reduced total neuroregeneration and not specifically new born motor neurons. Additional markers will be needed to be able to observe the entire ERG response such as sox2 immunohistochemistry. Alternatively,

new activator lines that use a different fluorophore could be generated. DsRed and mCherry retain signal strength after a lesion or can be enhanced with antibodies.

Dnhdac1 expression in the adult spinal cord did not reduce neuroregeneration or lesion induced proliferation of the ERGs. The founders for the *dnhdac1* line, though able to produce selective expression in the larval stages, gave reduced labelling in the adults compared with the double transgenic wildtype *hdac1* animals (compared in Figure 3.7). Therefore, a possible reason for why no significant reduction in the numbers of new born motor neurons was observed is because Hdac1 is not inhibited to the necessary extent. More founders are available who may give more robust induction in adulthood. The total number of PCNA positive cells is not a specific measurement of ERG proliferation. PCNA immunohistochemistry combined with a ERG marker may provide a more precise measurement.

5.3.3 Hdac1 overexpression does not increase neuroregeneration

As Hdac1 activity in the ERG is necessary for regeneration, a gain of function approach was used to augment the levels of Hdac1 during regeneration in the ERGs. The cell specific expression of wildtype *hdac1* in both larval and adult zebrafish during regeneration did not change the numbers of new born neurons after a lesion. Though a negative result, it shows that the expression of YFP proteins themselves do not inhibit regeneration confirming the specificity of the result with *dnhdac1* expression. The treatment with a HAT inhibitor EML425 was also used to examine the effects of deacetylation on regenerative neurogenesis. Similarly, to *hdac1* overexpression, EML425 treatment during regeneration did not change the numbers of new born motor neurons after a lesion. Motor neuron production has been boosted with different treatments before, e.g. pergolide to increase dopamine signalling (Ohnmacht *et al.*, 2016). This indicates that higher motor neuron production in this model is possible. However, the endogenous *hdac1* may already have reached a maximum level after a lesion meaning there are no targets in the cell still to be deacetylated. This could then explain why no significant difference in motor neuron regeneration is seen when deacetylation is increased. It is interesting to note that though both the overexpression of *hdac1* and EML425 treatment do not significantly change the number of new born neurons they both trend in the same direction towards increasing neuronal generation. More experiments using HuC immunohistochemistry will be informative, as A priori power analysis shows increasing the number to 63 animals per group would make it statistically significant. The timing of the deacetylation may be an important factor in the process of regeneration. Previous work in our group found that

hdac1 expression was increased at 14 dpl in adult zebrafish (Figure 5.1A) and another study found *hdac1* was increased at 3 dpl as well (Hui *et al.*, 2014). *Hdac1* expression is increased by 24 hours after a lesion in larval zebrafish (unpublished observations in Figure 5.1B). In this study doxycycline was administered immediately after the lesion to induce *hdac1* expression. An earlier doxycycline treatment to avoid any delays in induction or pre-treatment with the HAT inhibitor could be examined.

Surprisingly, the expression of *hdac1* in the ERGs trends towards reducing lesion induced proliferation in the larval zebrafish to the same rate as *dnhdac1* expression. The experiments are still underpowered, total pMN proliferation power is 0.525 and recombined cell proliferation requires more n number. However, the number of progenitors in mitosis remained unchanged after *hdac1* overexpression. Therefore, additional experimental repeats are necessary. In the adult spinal cord overexpression of *hdac1* did not change the lesion induced proliferation, though as mentioned previously, a more precise measurement with an ERG marker may find different results. If *hdac1* overexpression reduces proliferation, it would be similar to what was found in the zebrafish retina where mosaic expression of *hdac1* supressed BrdU labelling in retinal cells during development (Yamaguchi *et al.*, 2005). This result may demonstrate that the right level of *hdac1* expression is necessary for correct spinal cord ERG proliferation. Overexpression or reduced activity of Hdac1 may both lead to dysregulation of the cell cycle. Additional experiments would need to be carried out to discover how *hdac1* expression reduced progenitor proliferation but still leads to the same numbers of new born neurons after a lesion.

5.3.4 Additional markers are required to measure proliferation

The effect that the expression of either *dnhdac1* or *hdac1* constructs had on lesion induced proliferation was inconsistent between measurement techniques. The Tet activator line in this study did have an AmCyan tag which labels all the *Her4.1* positive ERGs. The AmCyan, however, was not of consistent strength after a lesion and cannot be enhanced with antibodies. It was used to measure proliferation after *hdac1* overexpression but not carried forward to the experiments with the *dnhdac1*. The limitation of the *Olig2*:DsRed transgenic line is that it cannot inform us precisely what is occurring due to it additionally labelling other cell types; motor neurons and oligodendrocytes. Therefore, to accurately measure proliferation additional markers or readouts are necessary. One possible alternative would be to use flow cytometry in combination with a nuclear marker like Hoechst. This technique can be used to

measure the amount of DNA in specific cell population and calculate the percentage of cells that are in the different cell cycle phases (Kim and Sederstrom, 2015).

5.3.5 Interaction between the immune system and Hdac1 activity in the ERG needs more investigation

The epistatic experiments performed in this study did not show any interaction between the immune response and Hdac1 activity in the ERG after the lesion. These experiments were only from one independent experiment as in a second round of animals the immune manipulations alone did not significantly change the numbers of new born motor neurons, so were excluded from analysis. Therefore, additional experiments need to be performed to fully address the interaction between the immune system and Hdac1 in the ERG. The *Mnx1*:RFP line used gives varied levels of regeneration, as evident by control values ranging from 0.8 to 3.7 cells. A different readout of regeneration such as using the numbers of HuC/EdU cells may yield more consistent results.

In summary, the cell type manipulations demonstrate that the expression of possible dominant negative Hdac1 in ERG reduced neuroregeneration in line with the previous results obtained with the pharmacological inhibitors. Increasing deacetylation with either with a HAT inhibitor or using Hdac1 overexpression was not sufficient to increase neuroregeneration.

Chapter 6 General Discussion

In the mammalian spinal cord, the progenitors display low regenerative potential after injury. They are unable to generate neurons to replace the ones lost in the injury. The progenitors are not unresponsive to the injury, however, as they are able to produce other cell types such as glia (Meletis *et al.*, 2008; Barnabé-Heider *et al.*, 2010). Zebrafish, in contrast to mammals, can replace lost neurons after a complete spinal cord transection (Reimer *et al.*, 2008). The progenitors in the zebrafish spinal cord have retained their neurogenic ability past development. It has been previously shown that the *Olig2*-positve ERGs in the ventricular progenitor zone are the spinal progenitor cells that generate motor neurons (Reimer *et al.*, 2009). Developmental signalling involved in neurogenesis such as hedgehog, notch, dopamine and serotonin are reactivated in response to spinal cord injury (Reimer *et al.*, 2009, 2013; Dias *et al.*, 2012; Barreiro-Iglesias *et al.*, 2015). The immune system is also necessary for this regenerative neurogenesis (Ohnmacht *et al.*, 2016; Tsarouchas *et al.*, 2018). The intrinsic changes within the spinal cord progenitors necessary for the process are unclear. Epigenetic modifications are mechanisms the cell can use to change gene expression in response to injury. Changes in epigenetic modifications could be a mechanism that facilitates the integration of all the extrinsic signals to lead to a neurogenic gene programme needed for successful regeneration.

6.1 Generation of a tool to manipulate Hdac1 in ERGs

The epigenetic modifier Hdac1 has been shown to positively regulate zebrafish neurogenesis during development (Cunliffe, 2004; Harrison *et al.*, 2011). During regeneration development mechanisms are often reactivated (Cardozo *et al.*, 2017). Therefore, this project investigated the role of Hdac1 in neuroregeneration after spinal cord injury. In chapter 3 of this thesis, I generated two new transgenic lines to manipulate Hdac1 levels in spinal cord ERGs. A cell type specific manipulation is necessary to properly study Hdac1 in regeneration for several reasons. There is disparity between the studies performed in mammalian systems which find that Hdac1 has both negative and positive role in neurogenesis. The *in vivo* studies often use pharmacological inhibitors to inhibit Hdac1 which will target all cells in the body. This could lead to observations that are resulting from multiple different actions of Hdac1 inhibition in a wide range of cell types. *In vitro* studies can be performed in a range of different cell lines which, though are cell specific, can be derived from different locations within the CNS. Progenitors from different CNS niches have different

properties. Progenitors located in regions of the brain are active and continuously produce neurons into adulthood. Progenitors in other regions like the spinal cord, however, are quiescent in adulthood. These cells could have different epigenetic modifications that may impact on the subsequent treatments. Hdac1 has different effects in different locations in the CNS, negatively regulating proliferation in the retina but positively in other brain regions. Therefore, results cannot be generalised to the entire CNS and are specific to the region examined.

Hdac1 has known roles in the function of the immune system. Therefore, treatment with HDAC inhibitors after injury will impact the response of the immune system which is known to be necessary for successful regeneration. HDAC inhibitors can be class specific, but often the pan-inhibitor TSA is used. This will inhibit all classes which may individually have different roles in the process. Some HDAC inhibitors have other targets in the cell which may additionally play roles in regeneration.

I used the Tet-On system to generate transgenic lines that had inducible expression of wildtype Hdac1 or dominant negative Hdac1 in ERGs. This system provides spatial and temporal control of gene expression that other expression methodologies lack. These transgenic lines are specific to spinal cord ERGs, very little expression is observed in mature neurons. The expression is induced by treatment with the tetracycline, doxycycline, with some leaky expression in larval stages. Expression of the transgenes can be observed from 2 hours post treatment in larval stages but can only be induced after a lesion in the adult spinal cord. The expression level of the transgenes is sufficient to mimic expression changes after a lesion. A major limitation of the study is that acetylation levels have not been found to change after the expression of the transgenes. More work needs to be performed to confirm that the expression of *dnhdac1* leads to inhibition of Hdac1 function.

6.2 Deacetylation in the unlesioned spinal cord promotes neurogenesis

In chapter 4, I found evidence that deacetylation is sufficient to stimulate the spinal cord ERGs to generate neurons in the absence of an injury. Hdac1 overexpression in the unlesioned larval spinal cord led to an increase in the number of ERGs in mitosis. HAT inhibition in the unlesioned larval spinal cord led to an increase in the numbers of new born motor neurons. This is the first study to experimentally increase neurogenesis in the unlesioned spinal cord. Further work (see below) is necessary to

confirm that deacetylation through Hdac1 overexpression is sufficient to trigger neurogenesis in the quiescent spinal cord.

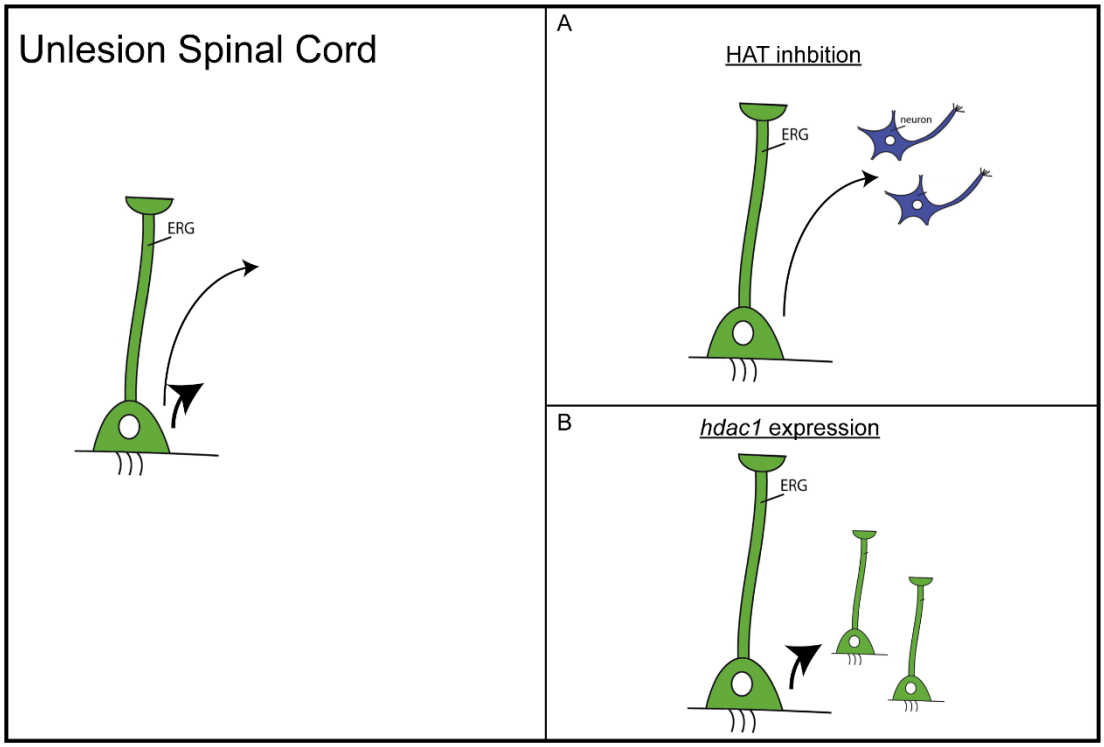


Figure 6-1:Summary of results in the unlesioned spinal cord. In the unlesioned spinal cord the ERG is relatively quiescent displaying low proliferation (short arrow) and neurogenesis (long arrow). **A**; After HAT inhibition neurogenesis was increased. **B**; After ERG specific *hdac1* expression proliferation was increased.

6.3 Hdac1 in ERGs is necessary for neuroregeneration in the lesioned zebrafish spinal cord

In chapter 5, the function of Hdac1 in the ERG during regeneration was examined. The expression of a putative dominant negative Hdac1 in the ERGs after spinal cord transection in larval zebrafish reduced the number of neurons generated. Both the numbers of new born motor neurons and total new born neurons were decreased. This reduction was found to be due to the reduction of the ERG lesion induced proliferation. Therefore, Hdac1 is a positive regulator of neuroregeneration in the zebrafish spinal cord. Increasing deacetylation, through Hdac1 overexpression in the ERG or global HAT inhibition, did not change the numbers of new born motor neurons after a lesion. More evidence is required to confirm the effect of *hdac1* expression on ERG lesion induced proliferation. Hence, the lesion induced changes in Hdac1 cannot be augmented further to lead to additional neurons being generated.

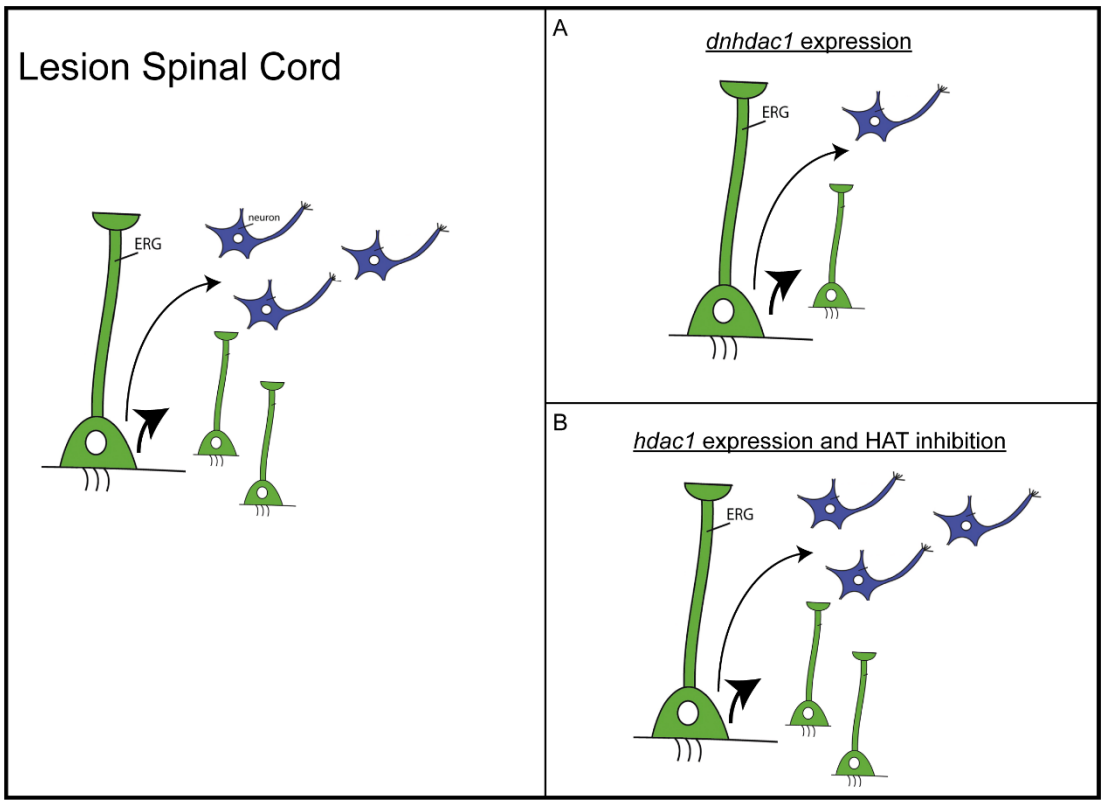


Figure 6-2: Summary of results in the lesioned spinal cord. After a lesion the ERG proliferates (short arrow) and produces new neurons (long arrow). **A**; ERG specific expression of *dnhdac1* reduced ERG proliferation and regenerative neurogenesis. **B**; Deacetylation through HAT inhibition or *hdac1* expression did not change regenerative neurogenesis and may reduce proliferation.

6.4 Future directions

The Tet-On system described in this thesis provides a new tool by which pathways can be tested for function in neuroregeneration in the spinal cord in a cell specific manner. A fundamental question in regenerative medicine is how the spinal cord progenitors in regenerating species give rise to new neurons to achieve successful repair after injury. Both nonregenerating and regenerating species display expression of extrinsic signals after injury, but the subsequent response of the progenitors is different. The molecular mechanisms of the extrinsic signals and how they converge remain unclear. Therefore, there is a need to study the intrinsic changes that occur within the progenitors after injury. Preliminary experiments in this study used the cell specific manipulations of Hdac1 to begin to test the mechanism for how extrinsic signals are integrated within the progenitors after injury. Further experiments using signals that promote neuroregeneration e.g. monoaminergic neurotransmitters and the immune system can be combined with the ERG specific manipulation of Hdac1. Another advantage of the cell specific manipulations possible with this new tool is that

regenerative neurogenesis can be specifically inhibited. I hypothesise that axons will still be able to cross the lesion site and form connections as normal. Therefore, this new tool could be used to test the contribution of new neurons generated by the lesion to functional recovery.

As well as aid investigations into the extrinsic signalling that modulate the ERGs behaviour the transgenic lines can be used to investigate the downstream gene changes that occur within the progenitor. A starting point would be to observe the effect of the Hdac1 manipulations on known candidate pathways such as notch and hedgehog. The working model for how Hdac1 may influence these pathways are shown in Figure 6.3. Hdac1 has been shown in development to inhibit notch target genes (Cunliffe, 2004) and to increase the hedgehog signalling through the deacetylation of Gli transcription factors (Canettieri *et al.*, 2010; Coni *et al.*, 2013). New targets of Hdac1 activity could be found with gene expression profiling to identify the Hdac1- regulated transcriptome in regenerating zebrafish similar to what was used to investigate the function of Hdac1 in developmental neurogenesis (Harrison *et al.*, 2011). Transcriptomes of progenitors after a lesion could be compared to progenitors after a lesion expressing *dnhdac1* to discover the gene programme that is necessary for regeneration. Transcriptomes of progenitors in the unlesioned spinal cord can be compared to progenitors after *hdac1* overexpression to discover the gene programme that facilitates the progenitors to move out of quiescence. FACs with the AmCyan channel is possible so the *Her4.1* Tet activator transgenic lines can be used to isolate the progenitors. This will allow the observation of the changes happening within our cell of interest. Due to the mosaicism of expression of the transgenes, selection of progenitors that are YFP positive (expressing the transgenes) and progenitors that are YFP negative from within the same animal, could be used to provide additional controls.

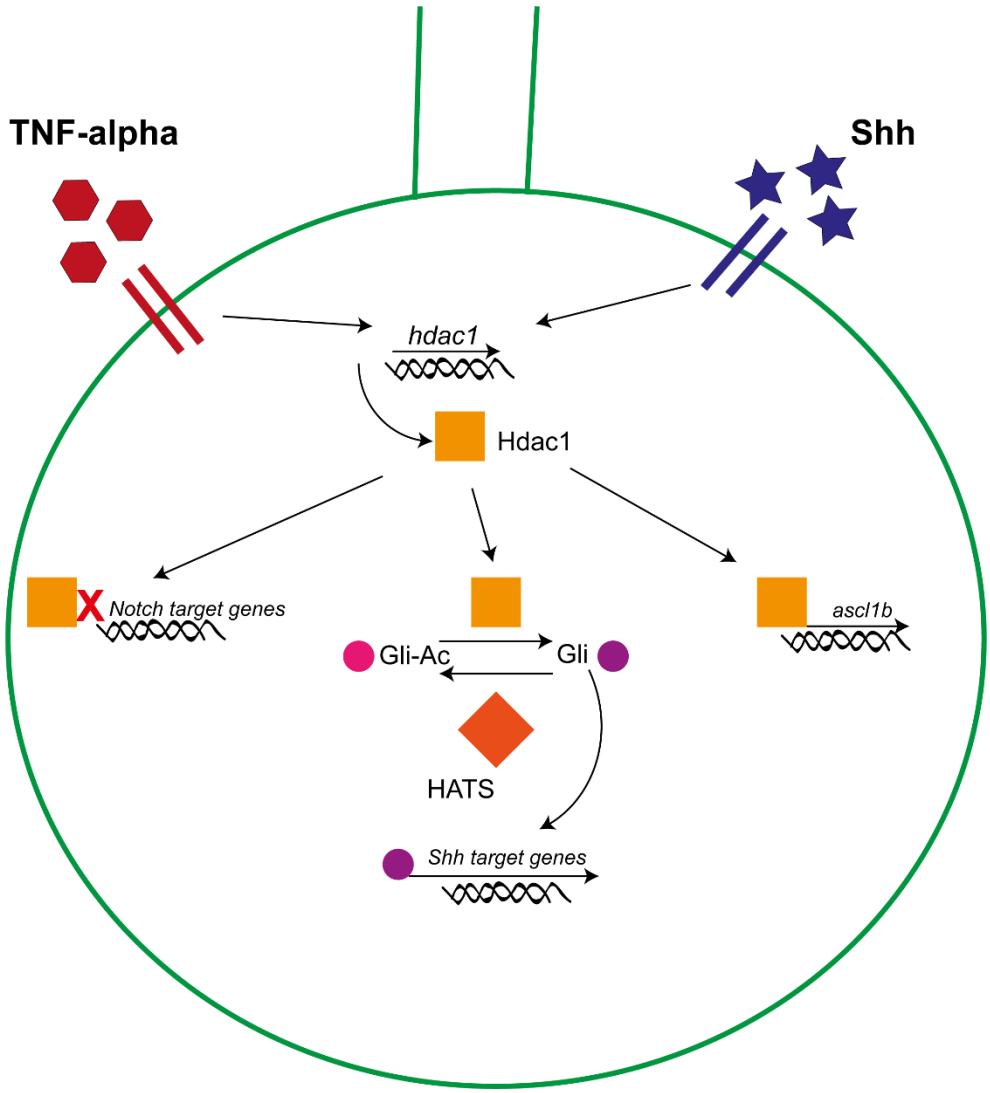


Figure 6-3: Schematic of the working model of the role of Hdac1 in controlling gene expression in the ERG. Extrinsic signals triggered by injury such as inflammation (e.g. TNF-alpha) and Shh lead to an increase in Hdac1 expression in the zebrafish spinal cord ERG. Hdac1 suppresses negative signals e.g. Notch target genes and amplifies positive signals directly e.g. ascl1b or indirectly through acetylation of other transcription factors e.g. Gli.

There is already evidence that not all ERGs are the same; some dorsal progenitor domains do not show the same regenerative potential that the pMN domain displays (Kuscha, Frazer, *et al.*, 2012; Ohnmacht *et al.*, 2016). Single cell sequencing could be a method to distinguish the different ERG populations to observe difference in gene expression between progenitors that respond to injury and ones that do not.

I have used larval and adult zebrafish to examine the role of epigenetic modifications specially in the ERGs during spinal cord regeneration *in vivo*. The insights we gain from these studies will inform knowledge on the mechanisms that enable successful

regeneration and help discover therapeutic strategies to promote regeneration in the mammalian spinal cord.

List of Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
6-OHDA	6-Hydroxydopamine
cAMP	cyclic adenosine monophosphate
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
ChAT	choline acetyl-transferase
CNS	central nervous system
Ctgfa	connective tissue growth factor a
CysLT1	cysteinyl leukotriene receptor 1
Cxcr5	C-X-C chemokine receptor type 5
DCX	doublecortin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpf	days post fertilisation
dpl	days post lesion
DsRed	Discosoma sp. Red fluorescent protein
EdU	5-ethynyl-2'-deoxyuridine
ERG	ependymal radial glia
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
Gli	Glioma- associated
HAT	Histone acetyltransferases
Hb9	homeobox protein 9
HDAC	Histone deacetylase
Her	hairy-related
hpf	hours post fertilisation
IL	interleukin
IHC	Immunohistochemistry
Irf8	interferon regulatory factor 8
Isl	Islet

LTC4	leukotriene 4
LPS	lipopolysaccharide
MBP	myelin basic protein
Mnx	motor neuron and pancreas homeobox
MS222	aminobenzoic acid ethylmethylester
mRNA	messenger RNA
NDS	normal donkey serum
NEB	new England biolabs
NeuN	neuronal nuclei
Ngn	neurogenin
NPA	R(-)-propylnorapomorphine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
pH3	phopho-Histone H3
PKA	Protein Kinase A
pMN	motor neuron progenitor
PNS	peripheral nervous system
ptch	patched
RA	retinoic acid
RFP	red fluorescent
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of mean
SGZ	subgranular zone
Sox	SRY-related HMG-box
SVZ	subventricular zone
TAE	Tris-acetate beuufer with EDTA
TNF-α	Tumour necrosis factor alpha
TSA	Trichostatin A
UAS	upstream activated sequence
VPA	Valproic acid

WIK	wildtype zebrafish strain
Wnt	wingless/Int

List of Figures

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